MODULATION OF ^{3}H-THYMIDINE INCORPORATION IN RAT LYMPHOCYTES BY ADRENERGIC DRUGS

BY

LAU LIT FUI

B.Sc. (Hons), C.U.H.K.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY

JUNE 1987

DEPARTMENT OF BIOCHEMISTRY

THE CHINESE UNIVERSITY OF HONG KONG

Acknowledgements

I would like to take this opportunity to express my most sincere gratitude to my supervisor, Dr. C.M. Lee. His expertise advice, harsh but constructive criticism, patience and friendly character have been most unforgettable during my two years of Master Program.

My special thanks are due to Dr. K.N. Leung for his advice on my cell culture techniques.

I also wish to express my heartful thanks to Mr. W.T. Cheung, Mr. S.O. Leung and Miss Y.W. Wong for their help and encouragement throughout the project.

Table of Contents

List of Abbreviations I

Abstract II

Chapter 1 : Introduction

1.1 Neuromodulation of Immunity 1

1.1.1 Adrenergic Action on Immune Responses

1.1.2 Adrenergic Receptors in The Immune System

1.1.3 Cholinergic Action on Immune Responses

1.1.4 Cholinergic Receptors in The Immune System

1.2 Lymphocyte Proliferation 6

1.2.1 Development of B Lymphocytes

1.2.2 Development of T Lymphocytes

1.2.3 Lymphocyte Growth Factors

1.2.4 Mitogens

1.2.4.1 Concanavalin A

1.2.4.2 Lipopolysaccharide

1.2.5 The Cell Cycle

1.2.6 ^{3}H-Thymidine Incorporation

1.3 The cAMP Signalling Pathway 15

1.3.1 Cyclic AMP and Lymphocyte Proliferation

1.3.2 Cyclic AMP and Con A

1.3.3 Cyclic AMP and LPS

1.3.4 Cyclic AMP and The Cell Cycle

1.4 The Phosphatidylinositol 4,5-Bisphosphate Pathway 20

1.4.1 Calcium and Lymphocyte Proliferation

1.4.2 Protein Kinase C and Lymphocyte Proliferation

1.4.3 Diacylglycerol, Phospholipids, cAMP and Cell Proliferation

Chapter 2 : Materials and Methods

2.1 Materials 28

2.2 Methods 33

2.2.1 Preparation of Spleen Lymphocytes

2.2.2 Cell Counting

2.2.3 Lysis of Red Blood Cells

2.2.4 Radioreceptor Assay

2.2.5 Stimulation of cAMP Production

2.2.6 cAMP Determination

2.2.7 Lymphocyte Culture

2.2.8 Statistics

Chapter 3 : Results

3.1 Characterization of The Lymphocyte Culture System 41

3.1.1 Effects of Con A and LPS on ^{3}H-Thymidine Incorporation

3.1.2 Cell Concentration Dependence of ^{3}H-Thymidine Incorporation

3.1.3 Time Dependence of ^{3}H-Thymidine Incorporation

3.2 Effects of Adrenergic and Cholinergic Drugs on ^{3}H-Thymidine Incorporation 47

3.2.1 Action of Adrenergic and Carbachol on ^{3}H-Thymidine Incorporation

3.2.2 Cytotoxicity of Adrenergic Drugs

3.2.3 Time Dependence of Adrenergic Drug Action on ^{3}H-

Thymidine Incorporation

3.3 Effects of cAMP Accumulation on ^{3}H-Thymidine Incorporation 59

3.3.1 Effects of cAMP Elevating Drugs on ^{3}H-Thymidine Incorporation

3.3.2 Cytotoxicity of The cAMP Elevating Drugs

3.3.3 Cyclic AMP Accumulation stimulated by Isoproterenol

3.3.4 Time Dependence of cAMP Elevating Drug Effect on ^{3}H-Thymidine Incorporation

3.4 Effects of A23187 and PMA on ^{3}H-Thymidine Incorporation 73

3.4.1 Effects of A23187 on ^{3}H-Thymidine Incorporation

3.4.2 Effects of PMA on ^{3}H-Thymidine Incorporation

3.4.3 Effects of Various Drugs on PMA-Induced ^{3}H-Thymidine Incorporation

3.5 Adrenergic and Cholinergic Receptor Binding on The Rat Spleen Lymphocytes 83

Chapter 4 : Discussion

4.1 Some Characteristics of The Lymphocyte Culture System 88

4.2 Effects of Adrenergic and Cholinergic Drugs on ^{3}H-Thymidine Incorporation 91

4.3 Effects of cAMP on ^{3}H-Thymidine Incorporation 95

4.4 Effects of A23187 and PMA on ^{3}H-Thymidine Incorporation 98

Conclusion 104

References 106

Appendix

List of Abbreviations

ATP	Adenosine triphosphate
BCGF	B cell growth factors
BMF	B cell mitogenic factor
BSA	Bovine serum albumin
C	Adenylate cyclase
cAMP	Cyclic adenosine 3',5'-monophosphate
cGMP	Cyclic guanosine 3',5'-monophosphate
Ci	Curie
con A	Concanavalin A
DG	Diacylglycerol
DHA	Dihydroalprenolol
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine tetraacetic acid
FCS	Fetal calf serum
G_i	Inhibitory guanine nucleotide protein
G_s	Stimulatory guanine nucleotide protein
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBMX	3-Isobutyl 1-methylxanthine
Ig	Immunoglobulin
Il	Interleukin
IP_3	Inositol 1,4,5-trisphosphate
IP_4	Inositol 1,3,4,5-tetrakisphosphate

LPS	Lipopolysaccharide
PAC	Para-aminoclonidine
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PIP_2	Phosphatidyinositol 4,5-bisphosphate
POPOP	1,2-Bis(2,5-phenyloxazolyl)benzene
PPO	2,5-Diphenyloxazole
PMA	Phorbol 12-myristate 13-acetate
QNB	1-Quinuclidinyl phenyl-benzilate
R_i	Inhibitory cell surface receptor
R_s	Stimulatory cell surface receptor
RNA	Ribonucleic acid
S.D.	Standard deviation
TdR	Thymidine

Abstract

The action of adrenergic drugs on Sprague-Dawley rat spleen lymphocyte proliferation in the absence and presence of mitogens was studied by *in vitro* ^{3}H-thymidine incorporation. When the incubation was carried out at 37 °C for 48 hours followed by 6 hours pulse labelling with ^{3}H-thymidine, optimal incorporation of the radiolabel can be obtained using 3 x 10^5 cells per well in the presence of either 1 ug/ml concanavalin A (con A) or 10 ug/ml lipopolysaccharide (LPS) or 10^6 cells per well in the absence of mitogens.

The adrenergic drugs used included isoproterenol, norepinephrine and epinephrine. They were found to exert an inhibitory effect on the basal, con A-induced and LPS-induced ^{3}H-thymidine incorporation. The IC_{50} were in the range of 10 uM to 100 uM. The specific binding of ^{3}H-DHA (1 nM) was estimated to be 2 fmole/10^6 cells, supporting the existence of beta adrenergic receptors on rat spleen lymphocytes. However, no $alpha_1$ and $alpha_2$ adrenergic receptors could be detected by radioreceptor assays using ^{3}H-WB4101 and ^{3}H-para-aminoclonidine as the respective radioligands. Furthermore, isoproterenol (1 uM) was able to stimulate cAMP accumulation in rat spleen lymphocytes from less than 1 pmole/10^6 cells to more than 6 pmoles/10^6 cells.

Dibutyryl cAMP which micmicked the action of cAMP and phosphodiesterase inhibitors including theophylline and isobutyl methylxanthine (IBMX) were able to suppress the basal, the con A-induced and LPS-induced ^{3}H-thymidine incorporation with IC_{50} ranging from 30 to 500 uM.

A23187, a calcium ionophore, exerted a biphasic action on the ^{3}H-thymidine incorporation in rat spleen lymphocytes. The optimal concentration for stimulation was cell concentration dependent, being 1 uM at 3 x 10^{5} cells per well and 300 nM at 10^{6} cells per well.

Phorbol myristate acetate (PMA), an activator of protein kinase C, was found to stimulate the basal ^{3}H-thymidine incorporation dose-dependently (0.1 - 1.0 ug/ml). Moreover, a biphasic action was observed on the LPS-induced ^{3}H-thymidine incorporation. The optimal stimulation occurred at 10 ng/ml PMA. On the other hand, it inhibited the con A-induced response. A synergistic action was also observed in the presence of 10 ng/ml PMA and 10 ug/ml LPS.

In the presence of PMA, dibutyryl cAMP (100 uM), theophylline (1 mM) and IBMX (100 uM) were still able to inhibit the basal, the con A-induced and the LPS-induced ^{3}H-thymidine incorporation. However, the three adrenergic drugs (100 uM) could only inhibit the basal and the LPS-induced but not the con A-induced response. This probably reflected a down regulation of the beta adrenergic signal tran[illegible]ction mechanism in the presence

of both con A and PMA.

In conclusion, it appears that the inhibitory effects of the adrenergic drugs on ^{3}H-thymidine incorporation in rat spleen lymphocytes might be mediated by an activation of the specific beta adrenergic receptor leading to an increase in cAMP accumulation. On the other hand, an elevated calcium level and an increase in protein kinase C activity are both stimulatory. Cyclic AMP produced by beta adrenergic activation may suppress lymphocyte proliferation by inhibiting either the protein kinase C, or the regulatory signals acting synergistically with protein kinase C, or the obligatory steps in DNA synthesis.

CHAPTER ONE

INTRODUCTION

1.1 Neuromodulation of Immunity

The immune system helps the proper functioning of an organism by fighting against foreign "invaders". The immune responses are, however, stringently controlled so that they are detrimentous only to foreign invaders but remain harmless to the host's own components.

Immune responses are regulated, to a large extent, by the immune system itself, involving a network of helper and suppressor T cells and their products, antibody feedback systems, idiotypic-anti-idiotypic network of interacting antibodies and other regulatory factors (Besedovsky et al., 1981). The immune responses are also under the control of the nervous and endocrine systems. For instance, various immunity parameters including spleen cell number, thymus cell number, splenic mitogen responsiveness, thymocyte mitogen responsiveness, antigen responsiveness, natural killer cell activity and macrophage suppressor cell activity are affected by either lesions in the anterior hypothalamus, hippocampus or amydala (Roszman et al., 1985). It is also well known that the psychological state can affect one's immunity. For instance, the response of men's lymphocytes to an activating agent declined significantly within a month or two after the deaths of their wives (Besedovsky et al., 1981). Students taking examinations were found to have a reduction in their helper T cell and natural killer cell activities (Marx, 1985). Moreover, classical conditioning is able to alter one's immune response (Ader et al., 1983) e.g. the

repeated association of a neutral conditioned stimulus with antigenic stimulation could increase antibody production when animals were later re-exposed to the conditioned stimulus alone.

Both the nervous and the endocrine systems are able to secrete chemical messengers (neurotransmitters or hormones) which upon interacting with their specific receptors in the target organs can affect their biological activites. Indeed, many such mediators including adrenocorticotropic hormone, bombesin, endorphins, enkephalins, neurotensin, substance P, somatostatin, vascactive intestinal peptide (O'Dorisio et al., 1985; Payan and Goetzl, 1985; Smith et al., 1985), arginine vasopressin, oxytocin (Johnson and Torres, 1985), insulin (Snow, 1985), prolactin (Russell et al., 1984), glucocorticoids (Ranelletti et al., 1987), serotonin (Jackson et al., 1985), norepinephrine and acetylcholine (see below) can exert their action on the immune system and/or have their respective receptors on the immune cells. Indeed, various lymphoid organs including the thymus, spleen, lymph nodes, gut-associated lymphoid tissue and bone marrow are innervated by the sympathetic noradrenergic nerve fibers and possibly peptidergic neurones as well (Felten et al., 1985). Such innervation is directed into regions of T lympocytes and the antibody producing plasma cells, but avoiding zones of developing B lymphocytes (Felten et al., 1985). Since surgical and chemical sympathectomy can increase the number of antibody forming cells, it has been suggested that the sympathetic nervous system may have a suppressive role on the immune system (Besedovsky et al., 1981; Felten et al., 1985). Furthermore, such

nerve innervation might even affect development and migration of cells into and out of the immune organs (Marx, 1985).

1.1.1 Adrenergic Action on Immune Responses

Local surgical denervation of the spleen as well as chemical sympathectomy by 6-hydroxy-dopamine followed by adrenalectomy have been shown to increase the number of antibody forming cells after immunization (Besedovsky et al., 1981). The norepinephrine content of the rat spleen decreases markedly on days 3 and 4 after the animal is challenged with sheep red blood cells. This decrease is more dramatic when the magnitude of the animal's immune response was high (del Rey et al., 1982). The turnover rate of norepinephrine is also lowered in the immunized rats (del Rey et al., 1982) reflecting a reduced synthesis and release of the biogenic amine. The norepinephrine level in the spleen of specific pathogen-free rats, which are constantly exposed to external antigens, is about half of that in the spleen of germ-free rats, which are exposed to a minimal degree of external antigenic challenge (del Rey et al., 1981). This association of low norepinephrine level in spleen with high immune response may be conveniently explained on the ground that norepinephrine is immunosuppressive. Thus, a decrease in norepinephrine may relieve the inhibition on the spleen cells and result in a higher immune response.

1.1.2 Adrenergic Receptors in The Immune System

The biological effects of norepinephrine and epinephrine are generally believed to occur as a result of the interaction of these catecholamines with specific receptors on the external surface of specific target cells (Minneman et al., 1981). Alquist (1948) proposed that there are two distinct types of adrenergic receptors, namely, the alpha and the beta adrenergic receptors. Alpha adrenergic activation is typified by smooth muscle contraction in which epinephrine is the most potent, with norepinephrine being equipotent or slightly less potent, and the synthetic amine isoproterenol being very weak. Beta adrenergic activation is typified by smooth muscle relaxation in which isoproterenol is the most potent, with epinephrine and norepinephrine being slightly weaker.

Alpha adrenergic receptor activation can increase the rate of E-rosette formation (T lymphocyte marker) (Xu and Chen, 1985) but inhibit the plaque forming cells and T-lymphocyte transformation (Besedovsky et al., 1979). On the other hand, beta adrenergic receptor activation can inhibit various B lymphocyte, T lymphocyte and macrophage functions e.g. lymphocyte mediated cytotoxicity, lymphocyte transformation and E-rosette formation. An increase in EAC-rosette (B lymphocyte marker) formation, however, has also been reported (Xu and Chen, 1985).

1.1.3 Cholinergic Action on Immune Responses

Cholinergic activation of lymphocytes is able to elicit an increase in intracellular cGMP level (Illiano et al.,1973) as well as an increase in RNA and protein synthesis (Hadden et al., 1975). Acetylcholine and carbachol, which are cholinergic agonists, can also depolarize the membranes of the T but not that of B lymphocytes (Shapiro and Strom, 1980). Immune parameters such as T-lymphocyte cytotoxicity (Strom et al., 1974), lymphocyte motility (Schreiner and Unanue, 1975), mitogen responsiveness (Illiano et al., 1973; Giliette et al., 1974; Richman and Arnason, 1979) and alloimmune-induced DNA synthesis (Strom et al., 1975) are all enhanced by cholinergic activation.

1.1.4 Cholinergic Receptors in The Immune System

Cholinergic receptors can be divided into the muscarinic and the nicotinic subtypes. The former can be activated by muscarine but inhibited by atropine. The latter can be activated by nicotine but inhibited by d-tubocurarine (Mayer, 1980). Since the various cholinergic responses described in section 1.1.3 are inhibited by atropine, they may be described as the muscarinic responses. In fact, by direct radioreceptor assay, the existence of muscarinic cholinergic receptors has been demonstrated in the lymphocytes of the rat (Shenkman et al., 1986) and human (Zalcman et al., 1981). Although Strom's group (1978) detected a muscarinic binding in the T but not the B lymphocytes, Atweh and

coworkers (1984) were able to detect the binding in both lymphocyte populations. Two types of muscarinic cholinergic receptors differing in binding affinities have also been reported (Richman and Arnason, 1979). Richman and Arnason (1979) also reported the existence of nicotinic receptors on lymphocytes which are able to inhibit proliferation upon activation.

1.2 Lymphocyte Proliferation

Of the various immune responses, lymphocyte proliferation is one of the central and basic requirement for the generation of immunity. Before one goes into the details of lymphocyte proliferation, it is useful to consider some aspects of the development of lymphocytes first.

1.2.1 Development of B Lymphocytes

B lymphocytes are responsible for the humoral antibody synthesis. They are developed in the Bursa of Fabricius in birds and hence the term, B lymphocytes. The equivalent of Bursa of Fabricius in mammals have not yet been identified but the most probable sources of B cells are the fetal liver and spleen as well as the bone marrow in adults (Roitt et al., 1985; Whitlock et al., 1985).

Murine B cell development can be divided into three phases

(Whitlock et al., 1985). Firstly, pluripotent haematopoietic stem cells are committed to the B cell pathway. They undergo immunoglobulin genes rearrangement and subsequent expression of IgM and IgD of the same specificity on their surface. Secondly, they have to migrate to and accumulate in peripheral lymphoid organs e.g. the spleen as resting B cells which can be activated by antigen and helper factors produced by T lymphocytes and accessory cells. Thirdly, the activated B cells can proliferate and differentiate into antibody producing plasma cells.

1.2.2 Development of T Lymphocytes

T lymphocytes are responsible for the cell mediated immune responses and they work in co-operation with B cells in the humoral antibody production. Precursors of T cells are produced in the bone marrow and they mature in the thymus under the influence of thymic hormones. Pre-T cells leave the bone marrow and migrate (Jotereau et al., 1980) to the thymus as a result of the concentration gradient of the thymic chemotactic factors. They then mature in the thymus as Lyt. 1+ and Lyt. 123+ cells which are known as the (T1) post-thymic precursor cells. These post-thymic precursor cells leave the thymus without passing through the thymic medulla and enter the periphery giving rise to T2 cell subsets : Lyt. 1+ helper T cells and Lyt. (1)23+ suppressor/cytotoxic T cells (Bach and Papiernik., 1981).

1.2.3 Lymphocyte Growth Factors

Lymphocyte proliferation is facilitated by soluble factors produced by immune cells. They include :

a) Interleukin 1 (Il-1), which is also known as the lymphocyte activating factor (Male, 1986). It is produced by mononuclear phagocytes when activated by antigens, lectins, lymphokines, phorbol esters, micro-organisms and various inflammatory agents (Gearing et al., 1985). It is able to induce interleukin 2 (Il-2) receptors on T cells (Male, 1986) and stimulate the production of Il-2 by T-cells (Smith, 1980).

b) Interleukin 2, which is originally known as the T cell growth factor. It is secreted mainly by the helper T cells when activated by lectins, antigens, calcium ionophores plus phorbol myristate acetate (Delia et al., 1984). Besides promoting T cell proliferation, Il-2 can also stimulate non T cell immune responses such as natural killer cell activity, lymphocyte activated killer cell activity and antibody secretion (Robb, 1984).

c) Interleukin 3 (Il-3) which is produced by lectin activated helper T cells and a number of leucocyte derived cell lines. Probably because of its ability to stimulate the proliferation of immature pluripotent cells, Il-3 has a wide range of biological activities many of which belong to the colony stimulating type

(Gearing et al., 1985).

d) B cell growth factors :

Besides being able to produce Il-2, activated helper T cells can also secrete two other lymphokines, namely the B cell growth factors (BCGF) I and II. They can stimulate activated B cells to proliferate. A third lymphokine, the B cell mitogenic factor (BMF) can cause the resting B cells to proliferate and to produce antibody. Fig. I is a simplified representation of the actions of various interleukins and B cell growth factors (Gearing et al., 1985).

1.2.4 Mitogens

Mitogens are molecules which can induce cell division and differentiation (Male, 1986). Mitogenic stimulation of lymphocytes in vitro is thought to mimic the series of events elicited by antigens on lymphocytes in vivo. Commonly used mitogens include concanavalin A (con A) and phytohaemagglutinin (PHA) which are specific for T lymphocytes and lipopolysaccharide (LPS) and dextran sulphate which are specific for B lymphocytes (Roitt et al., 1985). In my study, I employed con A as a T lymphocyte mitogen and LPS as a B lymphocyte mitogen.

1.2.4.1 Concanavalin A (Con A)

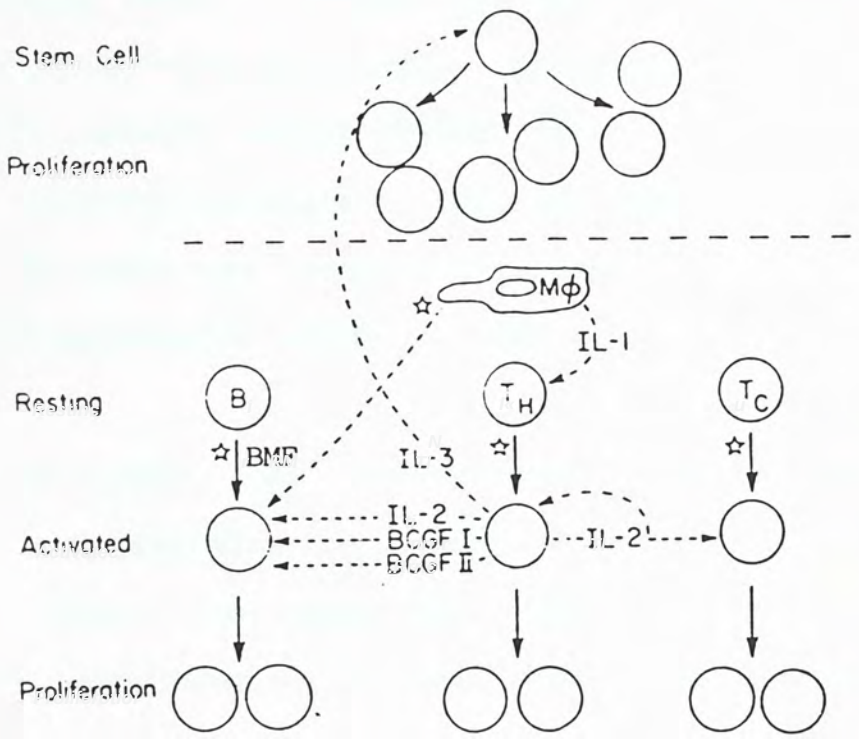

Fig. I Simplified diagram showing the involvement of the interleukins in leucocyte proliferation.
☆ represents antigen or lectin stimulation of monocytes (MΦ) or lymphocytes : cytotoxic/suppressor (T_C); helper (T_H).

Lectins are bivalent or polyvalent carbohydrate binding proteins (Goldstein et al., 1980). Among the various plant lectins, con A is the best characterized. It comprises up to 15 % of the proteins in the cotyledons of the jack bean (Canavalia ensiformis) seeds (Carrington et al., 1985). It carries no carbohydrate moiety and exists as a tetramer at pH 7.0-7.5 with a subunit molecular weight of about 25,500. Each subunit contains one saccharide binding site, one calcium ion and one manganese ion. The metal ions are probably responsible for maintaining the correct conformation of the molecule (Ling and Kay, 1975b).

Con A exerts its action by binding to the lymphocyte surface for a restricted time (Novogrodsky and Katchalski, 1971; Soren, 1973) without entering the cells (Greaves et al., 1972b). It binds specifically to the α-D-glucopyranosides, α-D-mannopyranosides, and polysaccharides or glycoproteins containing such residues (Summer et al., 1936). The saccharide binding activity depends on the calcium ion and manganese ion it contains (Yarvi et al., 1968). Since con A is multivalent, it can cross-link receptors between cells leading to the cell agglutinating phenomenon (Gunther et al., 1973).

The cellular reactions mediated by con A can be divided into the immediate versus the delayed type. The immediate type includes : con A receptor capping and patching; increase in glucose, nucleoside and potassium transport; increase in phospholipid synthesis; increase in cytosolic calcium, cAMP and

cGMP levels (Hadden et al., 1976). The delayed type reaction refers to the mitogenic action of con A on lymphocytes. Although both T and B lymphocytes bind a maximum of 10^7 con A molecules per cell, only T but not B lymphocytes exhibit a mitogenic response. Nevertheless, B lymphocytes may still be subjected to a certain degree of indirect stimulation from con A as a result of the diffused products of T lymphocytes (Ling and Kay, 1975c). Resting B lymphocytes can also be driven into a transitional activation state between the deep quiescence and G_o phase by con A (Hawrylowicz and Klaus, 1984).

1.2.4.2 Lipopolysaccharide (LPS)

LPS are endotoxins usually found in Gram negative bacteria and consist of a lipid component called lipid A and a polysaccharide component. Those produced by E. coli 0113 and 0111 : B4 are mitogenic for mouse and rat spleen cells (Ling and Kay, 1975b). Although they are able to bind to both T and B lymphocytes, they are only mitogenic for the B lymphocytes (Greaves and Baumdinger, 1972). Among the B lymphocyte population, only about 20 % is responsive (Janossy et al., 1973). LPS has also been shown to activate macrophage to secrete interleukin 1, interferons, and prostaglandins (Kurland et al., 1978) and to cause the release of factors from lymphoid cells which are mitogenic for thymocytes (Gery et al., 1972).

Upon incubation, LPS speeds up the phenotypic maturation of

pre-B lymphocytes and immature B lymphocytes to the mature stage. It also induces mature B lymphocytes to proliferate and differentiate into the antibody producing plasma cells (Andersson et al., 1972a, 1972b; Hammerling et al., 1976).

Like con A, LPS exerts its mitogenic effect without entering the cell (Greaves and Janossy, 1972). For the Coli and Salmonella LPS, the lipid portion of LPS is believed to be mitogenic (Andersson et al., 1973; Peavy et al. 1973; Rosenstreich et al., 1973). Lipid A probably interacts with the lipid bilayer of cell membrane to produce critical changes in the conformation of membrane proteins or other components to trigger off mitogenesis (Andersson et al., 1973).

1.2.5 The Cell Cycle

The eucaryotic cell cycle can be subdivided into four phases (Howard and Pelc, 1953; Barserga, 1981), i.e. the pre-DNA synthesis (G_1), the DNA synthesis (S), the post DNA synthesis (G_2) and the mitosis (M) phase. Following mitosis, the cell may either re-enter the cycle or enter the "resting" G_o phase in which the cell is not committed to cell division (Berridge, 1985). It is generally agreed that the S phase occupies half the time of a cycle while the remaining is split equally between G_1 and G_2 + mitosis (Marshall and Roberts, 1965; Sasaki and Norman, 1966). Nevertheless, variations exist in length of G_1, S, and G_2

phases (Steffen and Stolzman, 1969). For example, it was found that most lymphocytes entered their first period of DNA synthesis between 48 and 72 hours after phytohaemagglutinin (PHA) addition but some entered at 24 hours and others at more than 100 hours (Ling and Kay, 1975d).

The small lymphocyte is nearly dormant and proliferatively inactive. It has a highly condensed chromatin, a reduced nuclear matrix, poorly developed Golgi apparatus, few mitochondria and polysomes. The cell cycle genes are also turned off. After the addition of a mitogen like concanavalin A (con A), the chromatin de-condenses and early cell cycle genes are turned on. The nuclear matrix expands. The number of mitochondria also increases. Ribosomes are made and transported into the cytoplasm. With this new synthetic machinery in place, proteins and enzymes are made and transported into the nucleus to trigger off DNA replication (Whitfield et al., 1985).

1.2.6 ^{3}H-Thymidine Incorporation

Lymphocyte proliferation is accompanied by an enlargement of cells e.g. the blast cells. People used to count the number of enlarged cells in cultures as an indication of proliferation. Since proliferating cells can pick up ^{3}H-thymidine from the medium into their DNA, radiometric techniques can be used to measure the loss of radioactivity from the medium which reflects the degree of cell proliferation. A more commonly employed

method, however, is to measure directly the amount of ^{3}H-thymidine incorporated into the cell (Carr et al., 1972). This assay method has been claimed to be the most reproducible, quantitative and sensitive assay of lymphocyte proliferation (Oppenheim, 1969) and was used in my experiments to study lymphocyte proliferation.

1.3 The cAMP Signalling Pathway

The hormonally regulated adenylate cyclase system is composed of at least five types of components : the catalytic moiety of adenylate cyclase (C), the stimulatory (G_s) and the inhibitory (G_i) guanine nucleotide regulatory proteins, and the stimulatory (R_s) and the inhibitory (R_i) cell surface receptors. Both G_s and G_i are multimeric proteins consisting of α, β and γ subunits (Gilman, 1984; Helmreich and Pfeuffer, 1985).

The beta adrenergic receptor is a stimulatory (R_s) receptor which interacts with G_s resulting in an exchange of GTP for GDP on the α subunit of G_s. This is followed by the dissociation of G_s into GTP.α_s and $\beta_s\gamma_s$. GTP.α_s in turn activates C to give cAMP from ATP. A rise in intracellular cAMP level is thus to be expected with the binding of adrenergic agonists to beta receptors on the cell surface (Gilman, 1984; Helmreich and Pfeuffer, 1985) (see Fig. II).

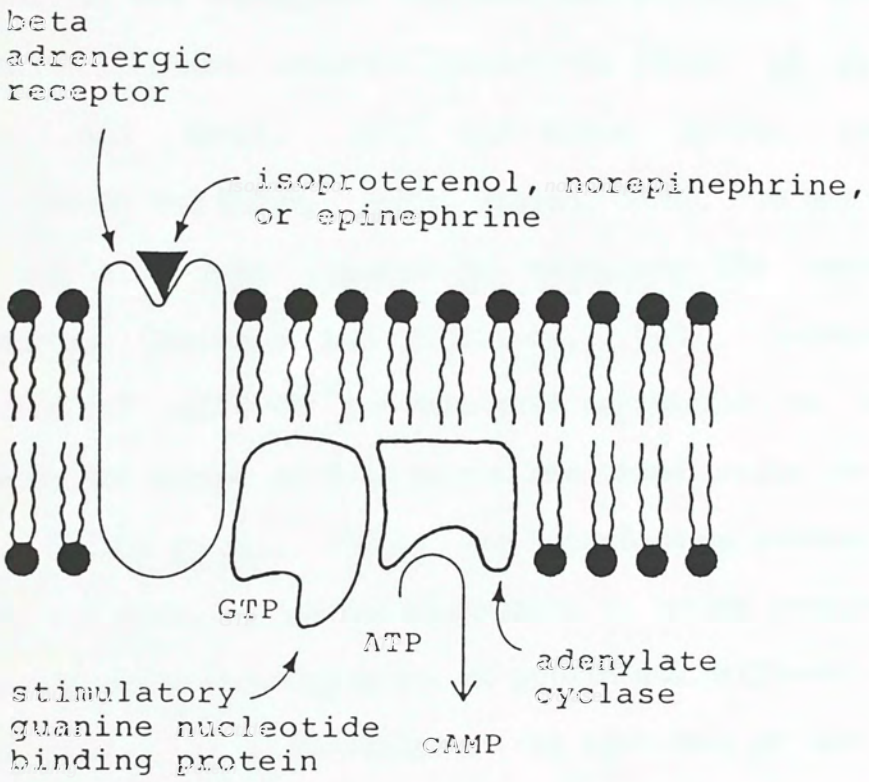

Fig. II <u>The beta adrenergic receptor linked adenylate cyclase system</u>

1.3.1 Cyclic AMP and Lymphocyte Proliferation

The action of cAMP on lymphocyte proliferation is two fold : both inhibitory and stimulatory effects have been observed.

Cyclic AMP and dibutyryl cAMP were found to inhibit DNA synthesis in rat thymocytes (MacManus and Whitfield, 1970), human lymphocytes, human leukemic lymphocytes (Abell et al., 1970; Johnson and Abell, 1970) and mouse spleen lymphocytes (Diamantstein and Ulmer, 1975; Watson, 1976). On the contrary, cAMP has also been reported to stimulate DNA synthesis in lymphocytes (MacManus and Whitfield, 1970). Intraperitoneal injection of cAMP or its dibutyryl derivative in rats also increases the number of bone marrow and thymus cells that enters mitosis (Rixon et al., 1970). The transforming protein produced by the ras gene, which may contribute to cancer development in malignant cells and regulation of growth and differentiation in normal cells, is an activator of the adenylate cyclase in yeast (Marx, 1984). All of these evidence argue for a positive role of cAMP in cell proliferation. Still other investigators could find no effect of cAMP on lymphocyte proliferation (Averner et al., 1972; Watson, 1975).

Such discrepancies may sometimes, although not always, be resolved by the fact that low concentrations of cAMP or its dibutyryl derivative (10^{-8} -10^{-6} M) stimulate DNA synthesis whereas high concentrations (between 10^{-4} -10^{-3} M) cause

inhibition (MacManus and Whitfield, 1970).

1.3.2 Cyclic AMP and Con A

Low concentration of con A which stimulates DNA synthesis has been found to inhibit adenylate cyclase activity in broken cell preparation of lymphocytes (Krug et al., 1972). However, at high concentration , con A inhibits DNA synthesis but stimulates adenylate cyclase activity. Similar observations have been made by other workers in intact human lymphocytes (Kaever and Resch, 1985; Hadden et al., 1976). Supraoptimal concentration of con A leads to a dramatic increase of cAMP level in the lymphocytes which may be responsible for the diminished DNA synthesis. Corresponding concentration of a con A derivative, succinyl con A, which did not increase cAMP also has no effect on the mitogenic response of lymphocytes. Cyclic AMP in these experiments was again assigned a negative role in lymphocyte proliferation. However, it should be noted that at optimal mitogenic con A concentration, cAMP did increase slightly (Kaever and Resch, 1985; Hadden et al., 1976). The large increase in cAMP induced by supraoptimal con A concentration may be due to the agglutinating properties of con A (Hadden et al., 1976) or the con A induced receptor capping phenomenon (Bourguignon and Hsing, 1983).

1.3.3 Cyclic AMP and LPS

Mitogenic concentrations of LPS cause little or no change in cAMP content in murine spleen cultures (Watson, 1975). More recently, the LPS mitogenic activity has been implicated to be mediated through the action of G_i , an inhibitory guanine nucleotide regulatory protein of the adenyate cyclase system (Jakway and DeFranco, 1986). For instance, pertussis toxin inhibits the LPS induced mitogenic responses of the WEHI-231 B_1 lymphoma cell line and the $P388D_1$ macrophage cell line. Since pertussis toxin can inactivate G_i through adenosine diphosphate ribosylation (Bockoch et al., 1983), it is possible that the LPS signal is transduced through a G_i receptor-effector coupling mechanism. Moreover, LPS has been demonstrated to inhibit adenylate cyclase activity in $P388D_1$ cells. LPS activation may thus decrease the intracellular cAMP level and relieve cAMP mediated inhibition of cellular responses (Jakway and DeFranco, 1986).

1.3.4 Cyclic AMP and The Cell Cycle

In the lymphocyte cell cycle, cAMP levels are highest in G_1 and G_2 phases. The levels at S phase and mitosis are only 20 % that of the G_2 phase (Millis et al., 1972).

It has been observed that the transition from G_o to G_1 is associated with a fall in cAMP (Otten et al., 1972; Frank, 1972).

Since an increase in cAMP level occurs within 15 min after the addition of mitogenic concentrations of PHA, it may be an early signal in the G_1 phase of lymphocytes (Krishnaraj and Talwar, 1973). However, other investigators have found that cAMP level in mouse spleen lymphocytes only rised 10 hours after exposure to mitogen, peaked around 20 hours, and then dropped before DNA synthesis peaked at 50 hours (Foker et al., 1979; Whitfield et al., 1980)

Cyclic AMP and protein kinase A have been postulated by Boynton and Whitfield (1983) to play a critical role in the pre-replicative events at the end of G_1 phase and in the pre-mitotic events. They also suggest that cAMP when generated at the right time at an appropriate level may promote cell cycle transit. However, it can block the cell cycle if generated at an abnormal amount at the wrong time.

1.4 The Phosphatidylinositol 4,5-Bisphosphate Pathway

In addition to changes in cAMP level, an elevated calcium ion level (Berridge, 1975; Metcalfe et al., 1980; Durham and Walton, 1982) and the activation of a neutral Na^+/H^+ exchange carrier (Moolenaar et al., 1981, 1982, 1983) may also contribute to the onset of cell proliferation. Since the phosphatidylinositol bisphosphate (PIP_2) signalling mechanism is involved in these biochemical changes (see below), it is not surprising that it has been a focus of research in lymphocyte

proliferation in recent years (Isakov et al., 1986).

The interaction of an external growth signal with the appropriate plasma membrane receptor can activate a PIP_2 specific phosphodiesterase, the phospholipase C, through a guanine nucleotide binding protein. Upon activation, it hydrolyses the plasma membrane PIP_2 to diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). These second messengers then act synergistically to elicit cellular responses in lymphocytes (Fig. III) (Mastro and Smith, 1983; Rozengurt, 1986). Con A has been found to enhance the PIP_2 turnover rate in lymphocytes (Sugiura and Waku, 1984) while no such change has been observed for LPS (Bijsterbosch et al., 1985).

1.4.1 Calcium and Lymphocyte Proliferation

Activation of the PIP_2 pathway can cause an initial transient release of calcium from an intracellular store and a more sustained entry from the extracellular space.

IP_3, one of the immediate product of PIP_2 hydrolysis, is able to release calcium from a non-mitochondrial intracellular store, probably the endoplasmic reticulum (Strab et al., 1983). IP_3 binds to a specific receptor (Spat et al., 1986) and opens a calcium channel (Muallem et al., 1985) on the endoplasmic reticulum through a guanine nucleotide regulatory mechanism (Gill

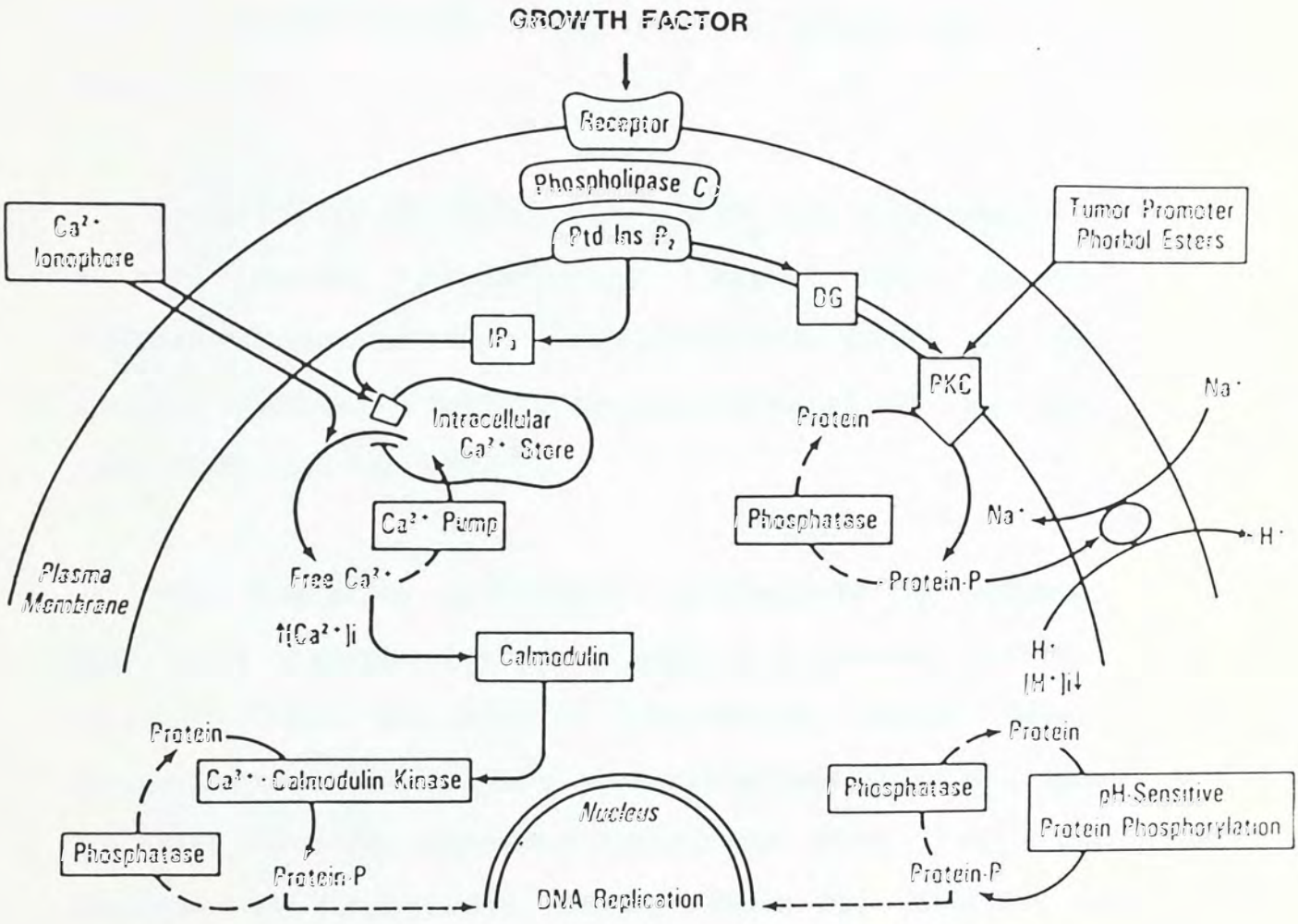

Fig. III Phosphatidylinositol 4,5-bisphosphate hydrolysis in lymphocyte proliferation

et al., 1986).

IP_3 can be phosphorylated to inositol 1,3,4,5-tetrakis-phosphate (IP_4) (Batty et al., 1985; Irvine et al., 1986) which can act synergistically with a fall in the intracellular calcium pool to provoke calcium entry from the extracellular space (Taylor, 1987).

To switch off the signal, IP_3 and IP_4 are dephosphorylated to give inositol 1,4-bisphosphate (Taylor, 1987). Eventual dephosphorylation of the inositol phosphate yields the myo-inositol which can be used for the resynthesis of PIP_2 (Berridge, 1984, 1985) (see Fig. IV).

When lymphocyte proliferation is stimulated by antigens, PHA, con A or calcium ionophore , there is a transient (0-6 hrs) small (0.1-1.0 uM) rise of intracellular calcium level. Extracellular calcium is required again between 19 and 47 hours to start chromosome replication (Mastro and Smith, 1982). The increase in intracellular calcium levels may directly or indirectly stimulate the transcription and translation of early genes in the cell cycle and increase the activity of their gene products. It also triggers the entry of replication enzymes and initiator protein(s) into the nucleus, mobilizes tubulin from the cytoskeleton/ to form the spindle, operates the spindle mechanism and triggers cytoplasmic division (Whitfield et al., 1985).

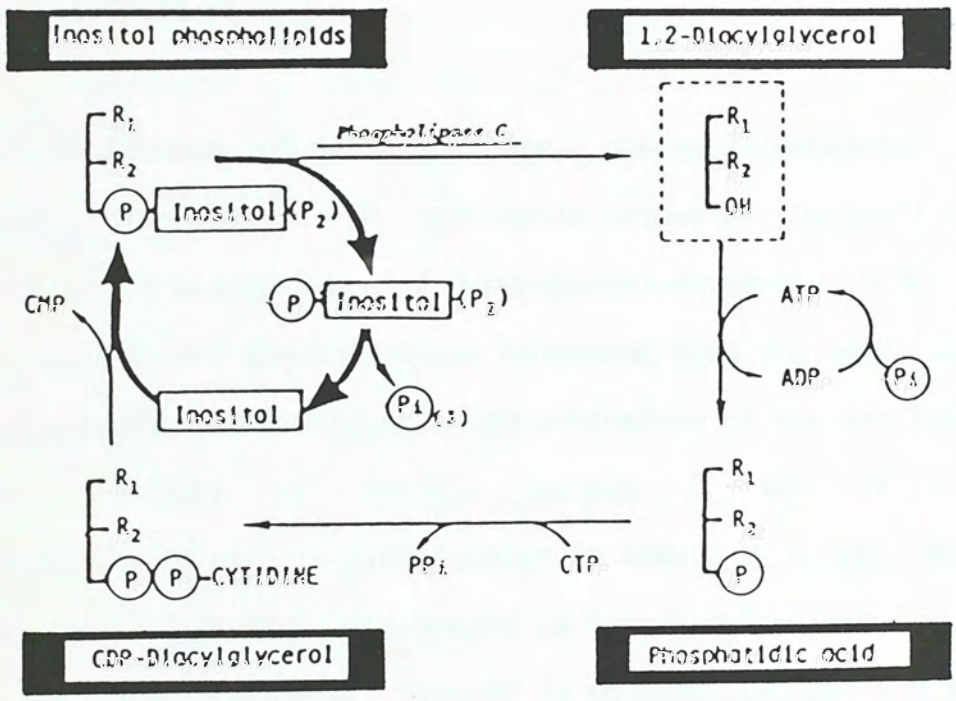

Fig. IV The turnover of phosphatidylinositol 4,5-bisphosphate

1.4.2 Protein Kinase C and Lymphocyte Proliferation

Protein kinase C was discovered in 1977 (Takai et al., 1977) and is widely distributed in many tissues. It is a phospholipid and calcium dependent serine/threonine protein kinase (Nishizuka, 1984a,1984b; Nishizuka et al., 1984). It comprises two functional domains : a regulatory hydrophobic domain which binds to the cell membrane upon activation and a hydrophilic domain which carries the catalytic site.

Activation of protein kinase C can be accomplished by DG, which is one of the PIP hydrolysis products. Derivatives of DG such as 1-oleoyl-2-acetyl-3-phosphoryl-glycerol (Nishizuka et al., 1984) and phorbol esters extracted from the seeds of Croton tiglium (Kolata, 1983) are also activators of protein kinase C. The affinity of protein kinase C for calcium and phosphatidylserine is dramatically increased by a small amount of DG. Upon activation, the enzyme is translocated from the cytosol to the plasma membrane. When DG is catabolized, protein kinase C is inactivated again. Alternatively, protein kinase C can be activated irreversibly by proteolysis although the physiological significance of such activation is not well understood (Murray et al., 1987).

The action of DG can be terminated by hydrolysis through diacylglycerol lipase to monoacylglycerol and fatty acid (usually arachidonic acid). DG can also be converted to phosphatidate by

diacylglycerol kinase for the resynthesis of PIP_2 (Berridge, 1984).

The Na^+/H^+ exchanger can be activated by phosphorylation through protein kinase C and thus exchanges extracellular Na^+ for intracellular protons resulting in an increase in intracellular Na^+ level and pH (Isakov et al., 1986). Other target proteins phosphorylated by protein kinase C include the IL-2 receptor (Shackelford and Trowbridge, 1984), guanylate cyclase (Zwiller et al., 1985), glucose transporter (Witters et al., 1985), tyrosine specific kinase (Hirata et al., 1984) and protein kinase C itself (Bell, 1986).

Like calcium mobilization, protein kinase C activation may be involved in the transcription of early genes in the cell cycle, translation of the gene transcripts produced and activation of the gene products as well as in mitosis and cytoplasmic division (Whitfield et al., 1985).

1.4.3 DG, Phospholipids, cAMP and Cell Proliferation

As mentioned above, arachidonic acid can be produced from DG by the action of diacyglycerol lipase. It can also be produced from phosphatidate by a phosphatidate specific phospholipase A_2 and from phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol by a less specific phospholipase. Arachidonic acid can then be converted to the E-type

prostaglandins which may leave the cell and stimulate cAMP production through specific prostaglandin receptors. The cAMP thus accumulated may play a role in the initiation of DNA synthesis in Swiss 3T3 cells (Rozengurt, 1986). Whether such a mechanism exists in the rat lymphocytes remains to be elucidated.

CHAPTER TWO

MATERIALS

&

METHODS

2.1 Materials

Experimental Animal

250-350 g male Sprague-Dawley rats supplied by the University Animal House, The Chinese University of Hong Kong were used throughout the experiments.

A23187

A23187 was purchased from Sigma. It was first dissolved in dimethylsulphoxide (DMSO) (Merck) to give a 10 mM stock and diluted with PBS to the appropriate concentrations.

Adrenergic drugs

The adrenergic drugs used include (-)-isoproterenol-bitartrate (Sigma), (-)-norepinephrine-HCl (Sigma), and (-)-epinephrine (Sigma). Drug solutions were freshly prepared before each experiment and made sterile by passing through a millipore filter (0.45 um).

Atropine

Atropine-sulphate purchased from Sigma was stored dessicated in the dark at room temperature. It was freshly prepared before each experiment.

Charcoal solution

Neutral activated charcoal was obtained from Sigma and prepared at a concentration of 75 mg/ml in the cAMP assay buffer containing 2 % BSA.

Complete medium

Under sterilized conditions, complete medium was prepared by adding 88 ml RPMI 1640 culture medium to 10 ml FCS, 1 ml PS and 1

ml fungizone to give a final concentration of 10 % FCS and 1 % of PS and fungizone.

^{3}H-Cyclic adenosine 3',5'-monophosphate (^{3}H-cAMP)

^{3}H-cAMP from Amersham with a specific activity of 23.6 Ci/mmole was freshly prepared each time at a concentration of 18 nM in the cAMP assay buffer.

cAMP standard

The stock concentration of cAMP standard (Amersham) was 16 pmoles/50ul. It was diluted with the cAMP assay buffer serially to give 8, 4, 2, 1 pmoles/50ul standard cAMP solutions.

cAMP assay buffer

The cAMP assay buffer contained 50 mM Tris-HCl and 4 mM Na EDTA at a pH of 7.4 at room temperature.

cAMP binding proteins

The cAMP binding protein was prepared according to the method of Brown et al. (1972). Bovine adrenal glands were freshly obtained from a local slaughter house. Fatty tissues on the surface were first trimmed off. The adrenal medulla was separated from the cortex and homogenized in 1.5 volume (v/w) of a solution containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.4 at room temperature. The homogenate was centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was collected and centrifuged at 10,000 x g for another 15 min. The supernatant thus obtained was divided into 500 ul aliquots and stored at -20 °C. Preparations of the cAMP binding protein was thawed and diluted 25 fold before used in the cAMP assay.

^{3}H-Dihydroalprenolol (^{3}H-DHA)

^{3}H-DHA having a specific activity of 55 Ci/mmole was obtained from Amersham and stored at -20 °C. Stock working solution was prepared at a concentration of 10 nM.

Ethylenediamine tetraacetic acid (EDTA)

Disodium salt of EDTA from Mallinckrodt was used in the cAMP assay buffer.

Dibutyryl cAMP

N ,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate was obtained from Sigma and stored at -20°C at 10 mM aliquots.

Fetal calf serum (FCS)

Fetal calf serum (Gibco) was stored in 10 ml aliquots at -20°C. The final concentration used was 10 % in RPMI medium.

Fungizone

Stock solutions of fungizone (300 ug/ml) was purchased from Gibco and stored in 5 ml aliquots at -20°C.

3-Isobutyl 1-methylxanthine (IBMX)

IBMX (Research Biochemicals Inc.) was first dissolved in absolute ethanol at 10 mM and then diluted to the appropriate concentrations with PBS.

Mitogens

Mitogens used included concanavalin A (con A) and E. coli. 0128:B12 lipopolysaccharide (LPS). Both were obtained from Sigma. They were dissolved in PBS at a concentration of 1 mg/ml and stored at -20 C in 0.3 ml aliquots.

^{3}H-Para-aminoclonidine (^{3}H-PAC)

^{3}H-PAC having a specific activity of 40 Ci/mmole was obtained from Amersham and diluted to a stock concentration of 10 nM for use in radioreceptor assays. It was stored at -20°C.

Penicillin-streptomycin (PS)

Stock penicillin-streptomycin solution containing 10,000 units/ml of pencillin G and 10,000 ug/ml of streptomycin sulfate was purchased from Gibco and stored in 5 ml aliquots at -20 °C. The final concentration used was 1 % of this stock solution.

Phorbol 12-myristate 13-acetate (PMA)

PMA was purchased from Sigma. It was first dissolved in DMSO (Merck) at 5mg/ml and diluted to the appropriate concentrations with PBS.

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 4 g NaCl, 0.1 g KCl, 0.57 g $Na_2HPO_4.12H_2O$ and 0.1 g KH_2PO_4 in 500 ml distilled water. The pH of the solution was then adjusted to 7.2 and made sterile by autoclaving at 121°C for 30 min.

1-Quinuclidinyl-(phenyl-4-^{3}H)-benzilate (^{3}H-QNB)

^{3}H-QNB (Amersham) with a specific activity of 38 Ci/mmole was stored at -20 °C. The stock concentration used in the radioreceptor assays was 20 nM.

RPMI 1640 culture medium

RPMI 1640 medium with 25 mM HEPES was purchased from Sigma in powdered form. Each bottle of RPMI powder was made up to one litre in distilled water. Two grams of sodium bicarbonate were added and the pH was then adjusted to 7.2. The solution was made sterile by passing through a millipore filter (0.45 um) under suction.

Scintillant

Scintillation fluid for radioreceptor assays contained 0.4 g

1,2-bis(2,5-phenyloxazolyl)benzene (POPOP) and 4 g 2,5-diphenyloxazole (PPO) in one litre mixture of 666 ml toluene and 333 ml Triton X-100 while that for ^{3}H-thymidine incorporation comprised of 0.125 g POPOP and 15 g PPO in 2.5 litres of toluene.

Theophylline

Theophylline was obtained from Sigma. It was used as a cAMP phosphodiesterase inhibitor.

^{3}H-Thymidine

^{3}H-Thymidine from Amersham with a specific activity of 2 Ci/mmole was diluted two fold with PBS to 0.5 Ci/ul for use in pulse labelling assay of lymphocyte proliferation. It was stored at 4°C.

Tris buffered isotonic ammonium chloride solution (Lysing Solution)

Ninety ml of 0.16 M ammonium chloride (BDH) solution was mixed with 10 ml of 0.17 M Tris-HCl (Sigma). The resulting solution was then adjusted to pH 7.2 with 1 M NaOH and sterilized by passing through a millipore filter (0.45 um).

Trypan blue solution

0.2 % trypan blue (Sigma) solution was prepared in PBS and used to check the viability of lymphocytes.

^{3}H-WB4101

^{3}H-WB4101 (Amersham) havin[illegible] activit[illegible] Ci/mmole was stored at -20°C. Stock working s[illegible]on was prepared at a concentration of 10 nM.

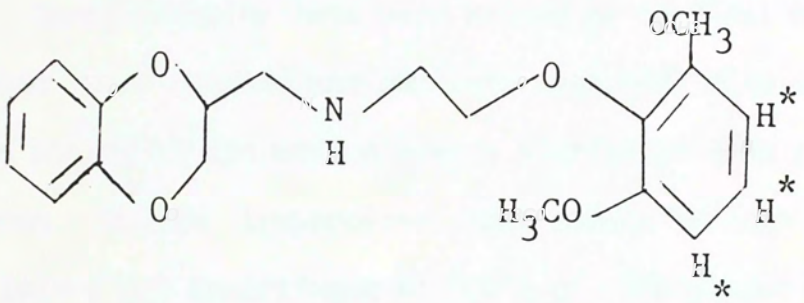

Fig. V Chemical structure of ^{3}H-WB4101

2.2 Methods

2.2.1 Preparation of Spleen Lymphocytes

Sprague-Dawley rats were killed by cervical dislocation. The spleen was removed and put into cold RPMI 1640 medium. It was then minced in the medium over a sterile 60 mesh stainless steel screen. Spleen lymphocytes that passed through the sieve were collected and centrifuged at 300 x g . The pellet was resuspended in 10 ml RPMI 1640 medium and centrifuged again at 300 x g. Finally, complete medium was used to resuspend the pellet and the cell concentration was adjusted to the required level (see below) after counting the number of cells with a haemacytometer.

2.2.2 Cell Counting

Twenty microliters of the cell suspension were diluted with 980 ul PBS. An aliquot of this diluted cell suspension (100 ul) was added to an equal volume of trypan blue solution and then applied to the haemacytometer. Since dead cells were stained blue in the trypan blue solution, they could be differentiated from the viable ones (Philip, 1973). The number of viable cells (y) was counted. The viable cell concentration of the original cell

suspension could then be calculated by the following equation :

$$y \times 100 \times 10^4$$

$$= 10^6 \, y \text{ cells/ml}$$

2.2.3 Lysis of Red Blood Cells (RBC)

In the case of radioreceptor binding and viability test, red blood cells (RBC) had to be lysed and removed first in order to avoid interference from them.

Every 0.1 ml cell pellet collected after the first centrifugation was resuspended in 1 ml of lysing solution at room temperature for 5 min in order to lyse the RBC. The spleen lymphocytes were then washed twice by centrifugation at 300 x g for 5 min. The cell pellet was finally resuspended in the complete medium unless otherwise specified.

2.2.4 Radioreceptor Assay

Four radioligands , ^{3}H-WB4101, ^{3}H-PAC, ^{3}H-DHA and ^{3}H-QNB were used to test the existence of alpha$_1$ adrenergic, alpha$_2$ adrenergic, beta adrenergic, and muscarinic cholinergic receptors on the rat spleen lymphocytes respectively.

The rat spleen lymphocytes without RBC were prepared as

described earlier except that PBS was used as the medium. The binding studies were similar for all three radioligands. Essentially, 50 ul of the radioligand, 50 ul of the displacer or buffer and 400 ul of the cell suspension were mixed together and incubated at an indicated temperature for an indicated period of time to ensure an equilibrium of binding (see Table I). The mixture was vortexed from time to time to prevent the cells from sedimenting under gravity. At the end of the incubation, the mixture was filtered through Whatman GF/B glass fibre filter under suction. The filters were washed 5 times using 5 x 3 ml cold PBS.

Nonspecific binding is defined as binding in the presence of an excess of displacer. By subtracting non-specific binding from total binding, one can obtain the specific binding for each radioligand.

2.2.5 Stimulation of cAMP Production

Rat spleen lymphocytes were prepared as previously described except that PBS, instead of RPMI 1640 medium, was used as the buffer medium throughout the experiment.

The spleen lymphocyte preparation was adjusted to a cell concentration of 3 x 10^7/ml and preincubated at 37°C for 10 min. To initiate cAMP accumulation, an aliquot (50 ul) of cell

Table I Conditions for radioreceptor assay

Radioligand		Incubation		Displacer
Name	Concentration (nM)	Time (min)	Temperature (°C)	
^{3}H-DHA	1	30	37	propanolol
^{3}H-WB4101	1	20	25	norepinephrine
^{3}H-PAC	1	30	25	norepinephrine
^{3}H-QNB	2	60	37	atropine

suspension was added to the 450 ul PBS containing various concentrations of isoproterenol and 12.5 mM theophylline. At the end of an indicated period of incubation, the reaction was stopped by boiling for 4 min. The precipitate was then centrifuged at 10,000 x g for 15 min at room temperature. Duplicate aliquots of the supernatant (50 ul) were used for cAMP determination.

2.2.6 Cyclic AMP Determination

The cAMP determination was based on the cAMP assay kit from Amersham. It depends on the competition for specific binding to the cAMP binding protein by radiolabelled and unlabelled cAMP. When there is more cAMP in the unknown sample, less ^{3}H-cAMP will be bound to the binding proteins and vice versa. With the introduction of activated charcoal, unbound ^{3}H-cAMP is adsorbed and can be removed by centrifugation. The amount of ^{3}H-cAMP bound to the binding proteins in the supernatant can be determined. This allows the determination of the amount of cAMP in the unknown sample by comparison with a standard curve.

Various agents were added into 1.5 ml microfuge tubes as shown in Table II. Each tube contained 200 ul reagents and was incubated at 4 C for at least 2 hours but not more that 16 hours.

Tube	Assay buffer	Standard cAMP	Sample	^{3}H-cAMP	Binding protein
Blank	150 ul	-	-	50 ul	-
Standard zero	50 ul	-	-	50 ul	100 ul
Standard	-	50 ul	-	50 ul	100 ul
Samples	-	-	50 ul	50 ul	100 ul

Table II cAMP determination assay

At the end of the incubation, an aliquot (100 ul) of an ice-cold charcoal solution, which had been kept stirred for at least 15 min before use, was added into each tube and mixed. Between 1 and 2 min after the addition of charcoal, the tubes were centrifuged at 10,000 x g for 2 min at 4°C. An aliquot of the supernatant (200 ul) was counted by liquid scintillation spectrometry.

The blank cpm, which was always a few hundred counts per min, was first subtracted from each datum. The specific binding in the absence of unlabelled cAMP standard (zero standard) was designated as Co, while that in the presence of unlabelled cAMP was designated as Cx. The ratio of Co/Cx could then be calculated and a standard cAMP calibration curve can be obtained (Fig. V).

2.2.7 Lymphocyte Culture

Lymphocytes were prepared as described in section 2.2.1.

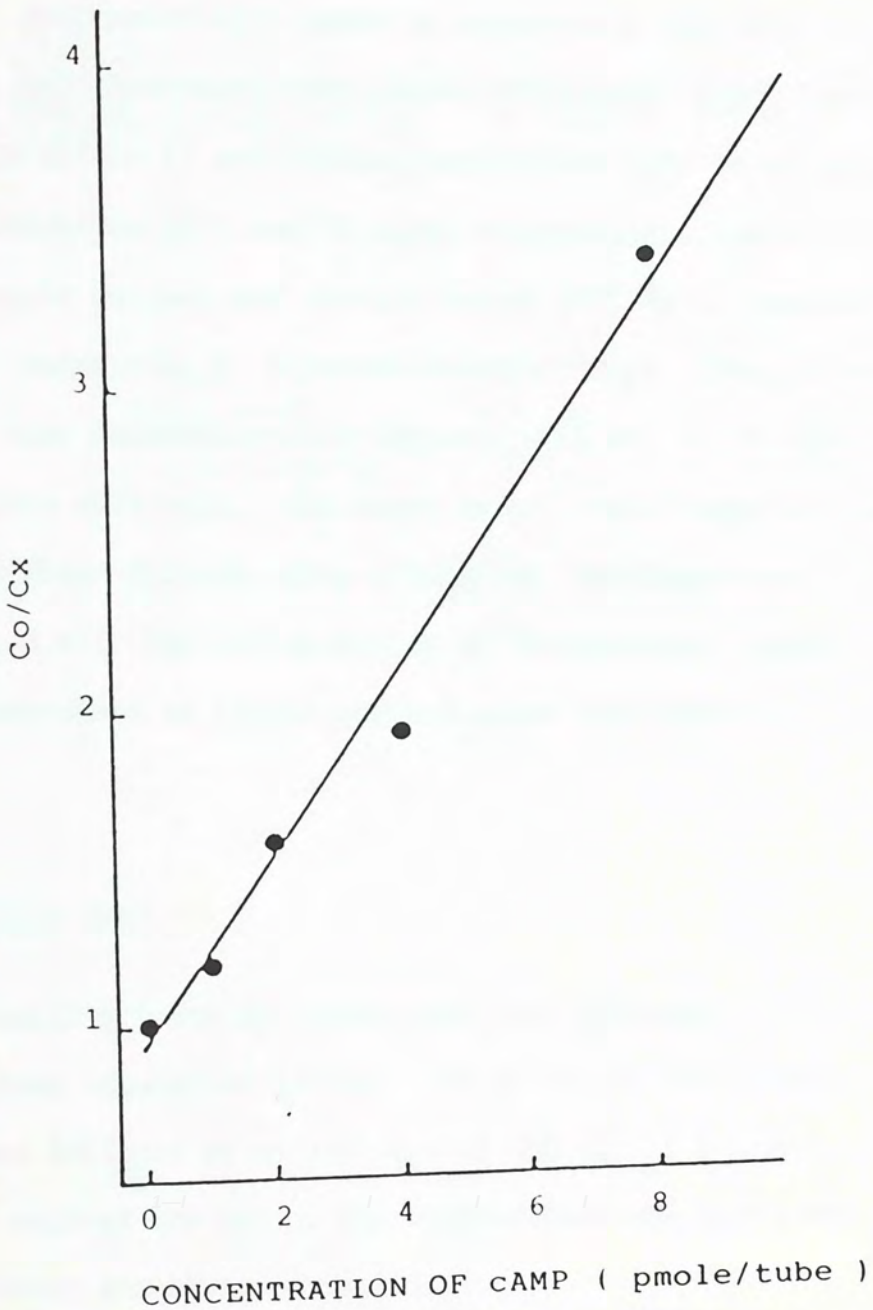

Fig. V A typical calibration curve for cAMP determination.

Values presented are the means of duplicated determinations from one typical experiment. The straight line was determined by linear regression analysis. Each experiment was accompanied by an individual calibration curve. Co and Cx are the binding of ^{3}H-cAMP in the absence and presence of unlabelled cAMP respectively.

Usually 50 ul of the drugs to be tested, 50 ul of the complete medium or mitogens in the complete medium and 100 ul of the lymphocyte suspension were added in sequence to the wells of 96-well flat bottomed microtiter plates. Mitogens used included concanavalin A (con A) and lipopolysaccharide (LPS) at an optimal final concentration of 1 and 10 ug/ml respectively. Incubation of the lymphocyte culture was carried out at 37°C in a humidified atmosphere containing 5 % carbon dioxide (CO_2). After 48 hours (except in time dependency experiments), 0.5 uCi of ^{3}H-thymidine was added into each well. Six hours later, cells were harvested onto glass fiber filters using a Titertek multiharvester (Flow Lab. Ltd., U.K.). The radioactivity of ^{3}H-thymidine incorporated was then determined by liquid scintillation spectrometry.

2.2.8 Viability Test

The viability tests on lymphocytes were performed at the end of the 48 hour incubation period. 100 ul of the supernatant was removed first followed by an addition of 100 ul 0.2 % trypan blue solution to each of the wells. The lymphocytes were then added to a haemacytometer for assay of viability.

2.2.8 Statistics

All results were expressed as the arithmetic mean $\pm$ standard deviation. The significance of difference between control and test groups was assessed by the Student's t test.

CHAPTER THREE

RESULTS

3.1 Characterization of The Lymphocyte Culture System

3.1.1 Effects of Con A and LPS on ^{3}H-Thymidine Incorporation

At low concentrations, con A dose-dependently stimulated ^{3}H-thymidine incorporation into the rat spleen lymphocytes (Fig. 1). However, at concentrations higher than 1 ug/ml the stimulated response diminished in a dose-dependent manner. Unlike the biphasic response to con A, ^{3}H-thymidine incorporation was increased monophasically by increasing concentration of LPS reaching a plateau at about 10 ug/ml (Fig. 2). Thus, the optimal doses of con A and LPS at 1 and 10 ug/ml respectively were used in all subsequent experiments.

3.1.2 Cell Concentration Dependence of ^{3}H-Thymidine Incorporation

In the absence of con A or LPS, ^{3}H-thymidine incorporation increased almost linearly as the number of cells per well (Fig. 3). In the presence of con A, ^{3}H-thymidine incorporation peaked at 5×10^{5} cells per well but dropped off rapidly at higher cell concentrations (Fig. 4). In the presence of LPS, ^{3}H-thymidine incorporation reached a plateau leveled at 10^{6} cells per well (Fig. 5). Unless otherwise specified, 10^{6} cells per well was chosen for study in all subsequent experiments in the absence of mitogens whereas 3×10^{5} cells per well was chosen for experiments with con A or LPS.

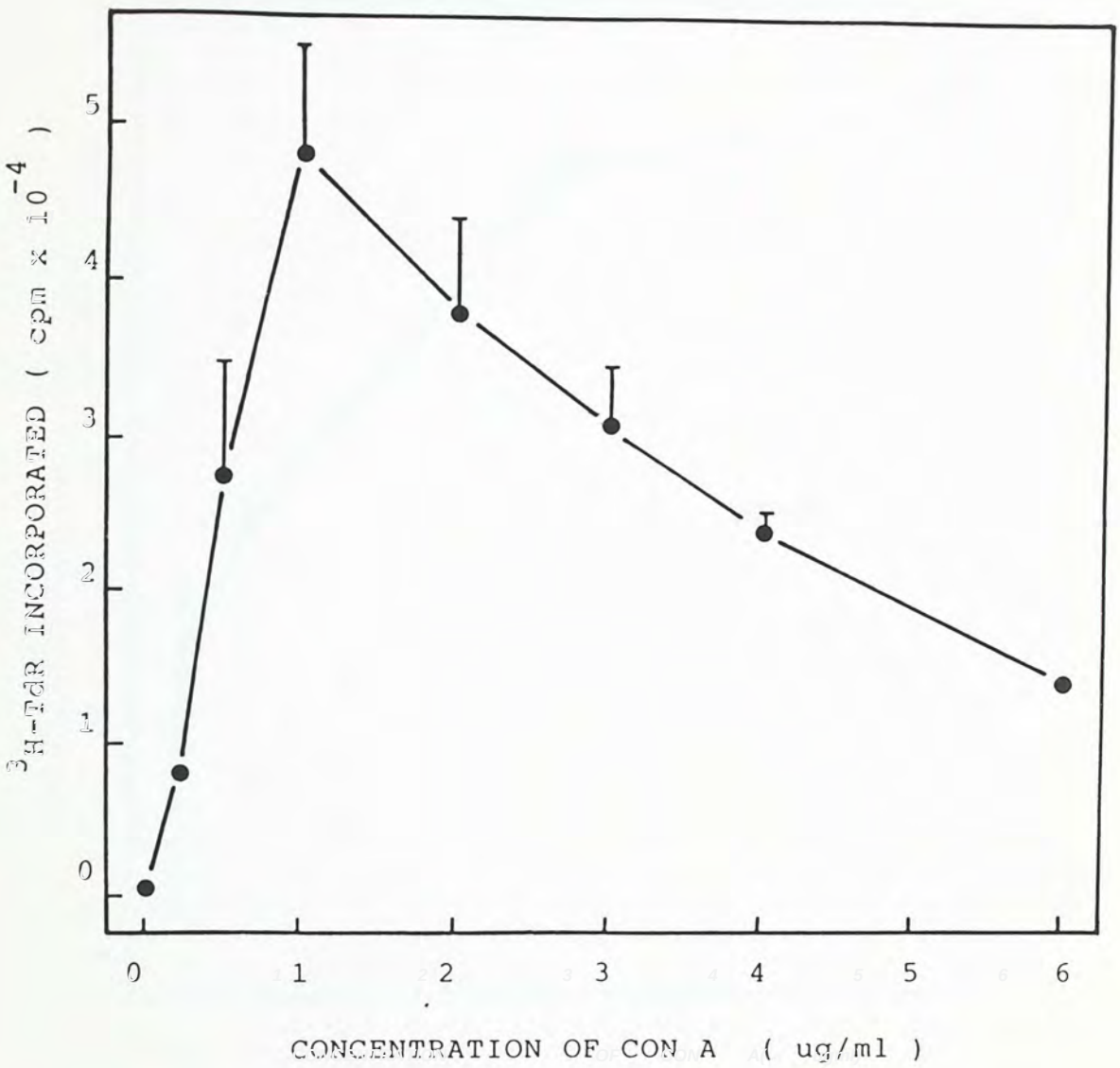

Fig. 1 Dose response curve of con A-induced ^{3}H-thymidine incorporation

3 x 10^5 rat spleen lymphocytes were cultured with different concentrations of con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

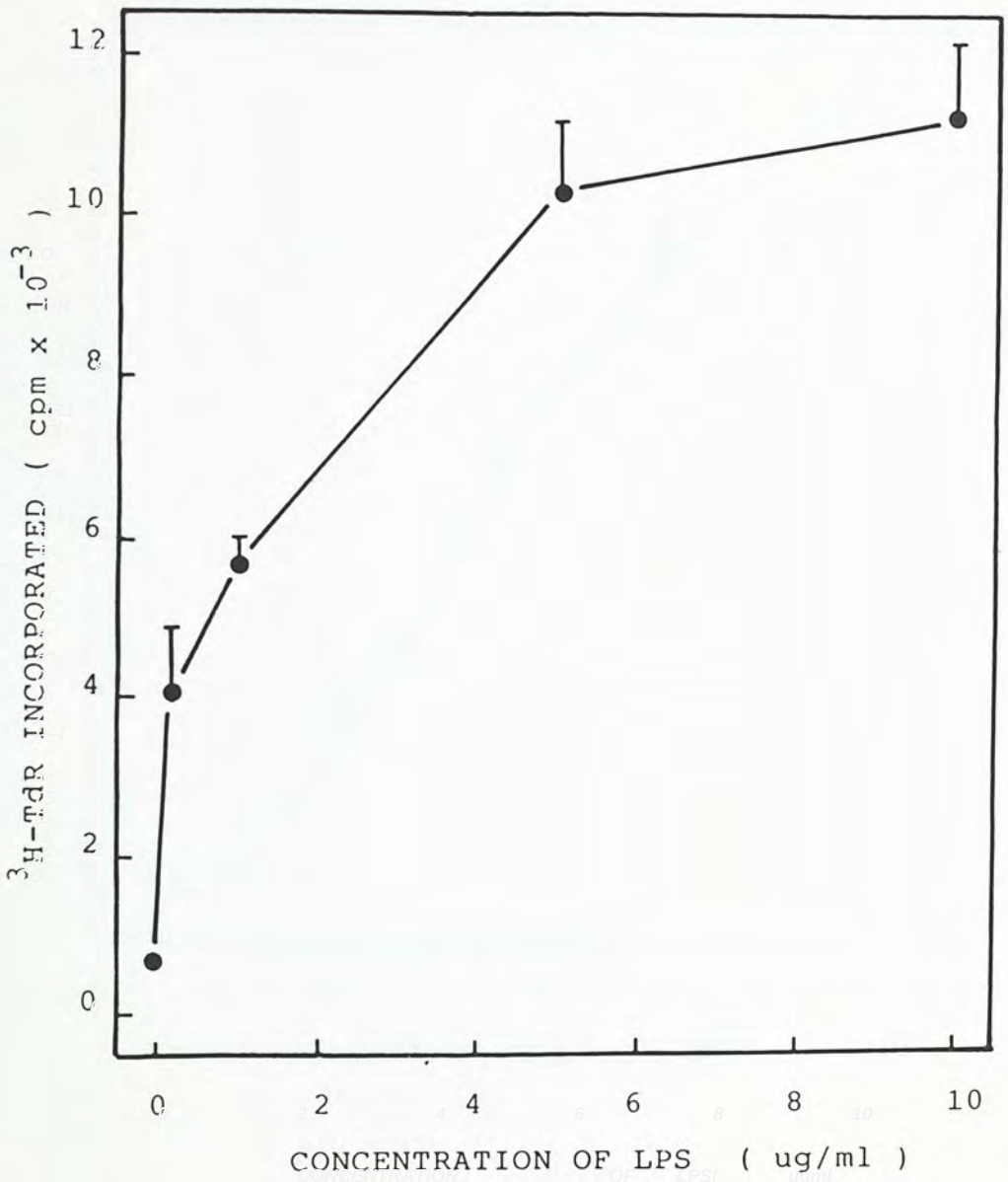

Fig. 2 Dose response curve of LPS-induced ^{3}H-thymidine incorporation

3×10^5 rat spleen lymphocytes were cultured with different concentrations of LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

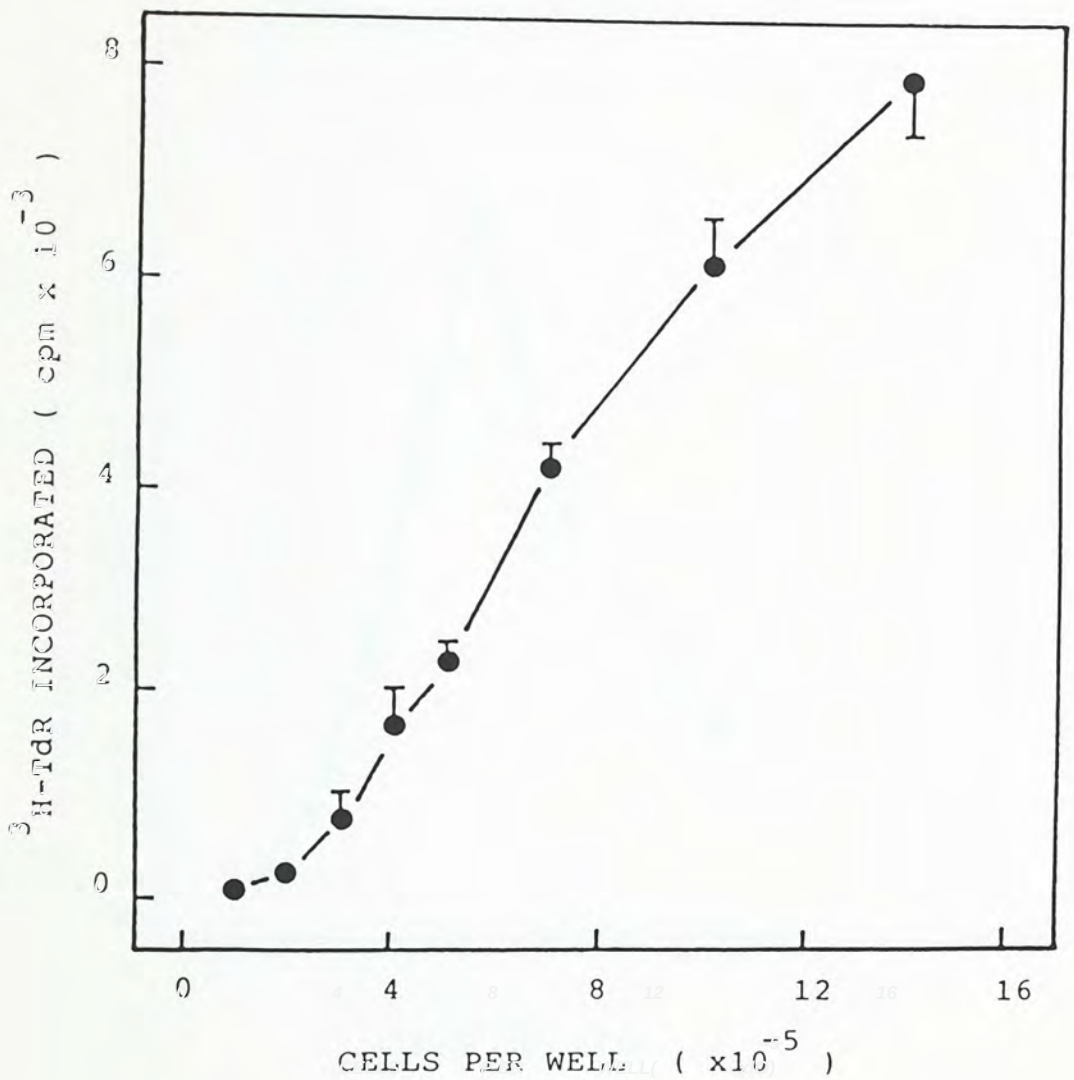

Fig. 3 Cell concentration dependence of the basal ^{3}H-thymidine incorporation

Various cell concentrations of rat spleen lymphocytes were cultured in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in two separate experiments.

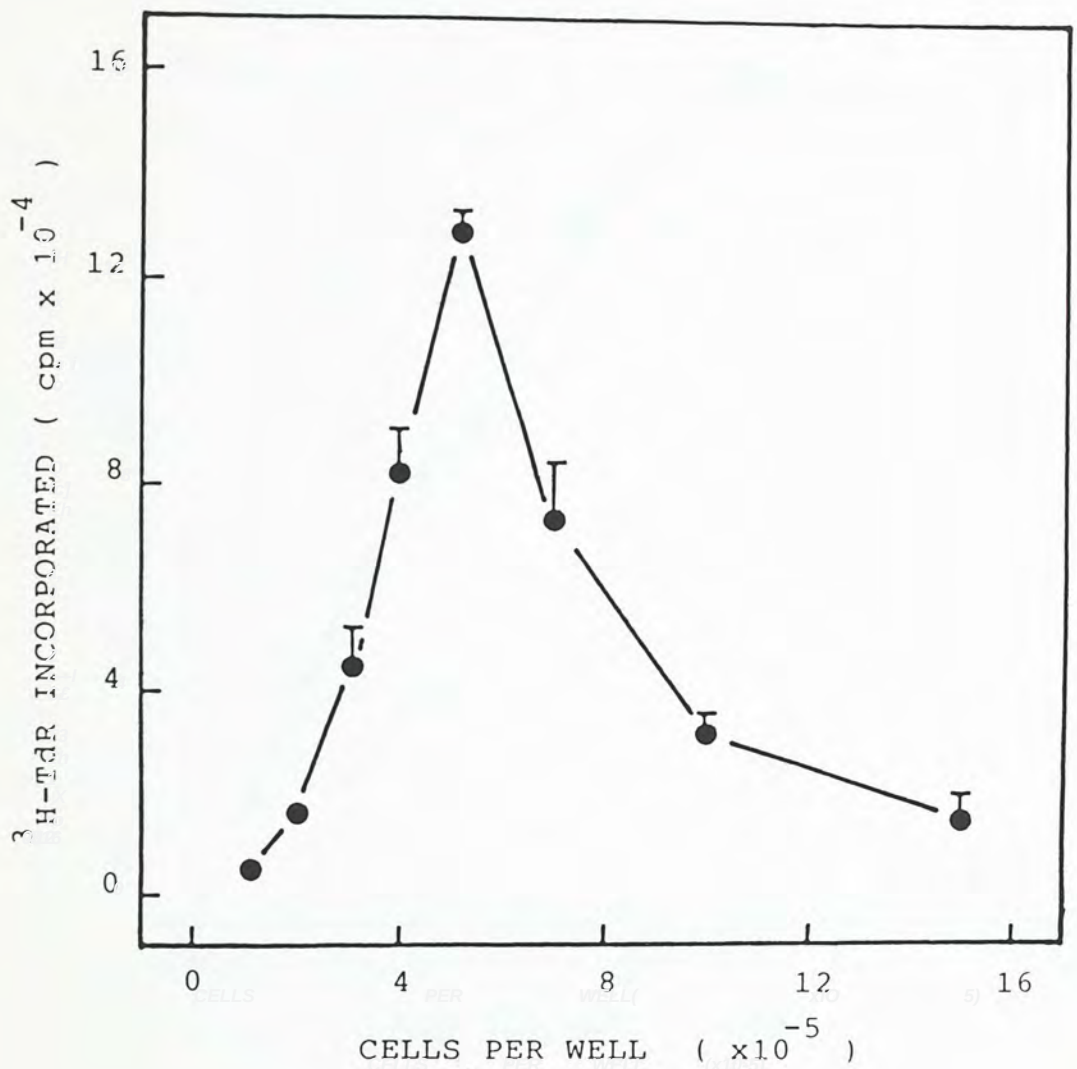

Fig. 4 Cell concentration dependence of ^{3}H-thymidine incorporation in the presence of con A

Various cell concentrations of rat spleen lymphocytes were cultured with 1 ug/ml of con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

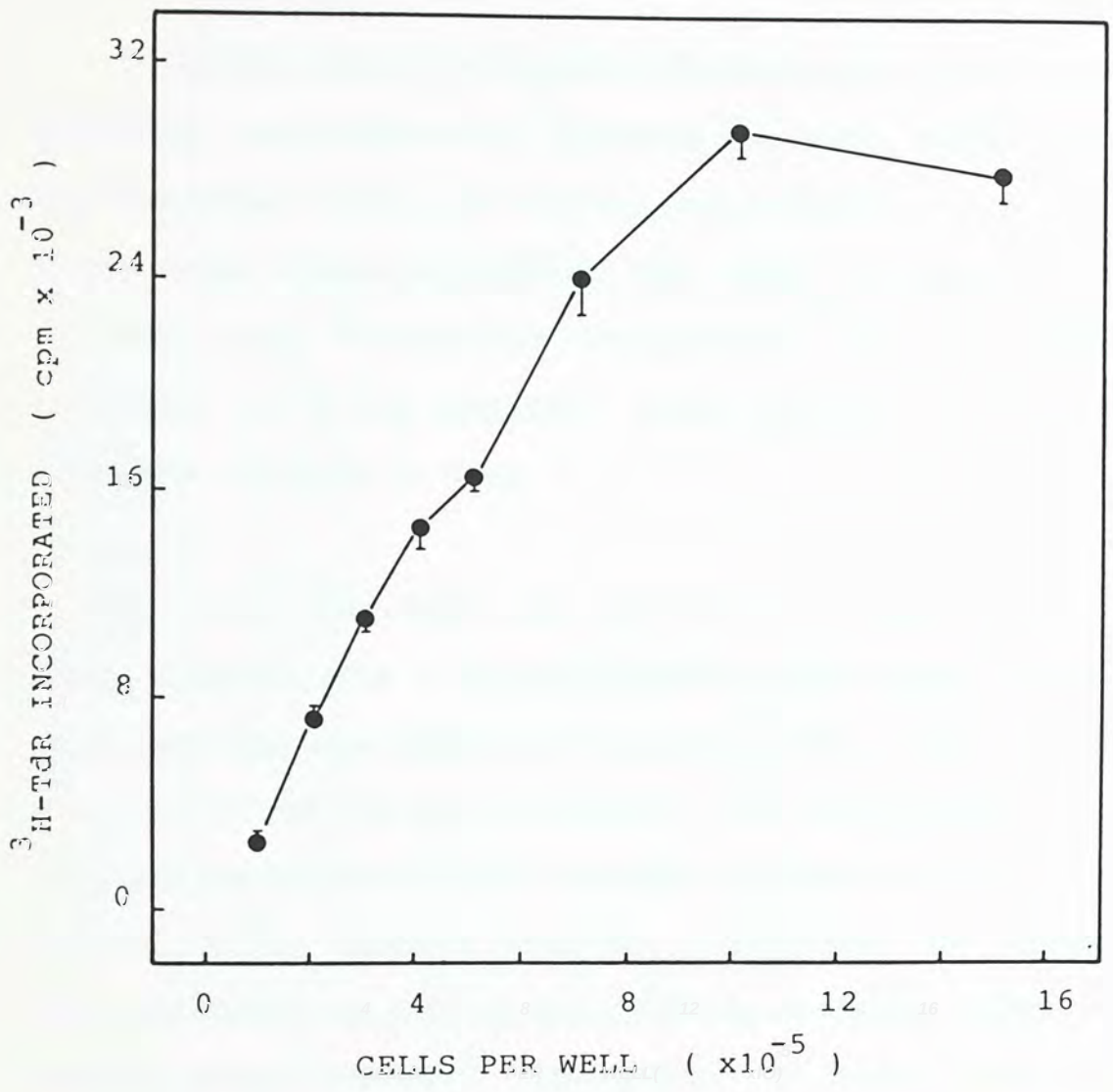

Fig. 5 Cell concentration dependence of ^{3}H-thymidine incorporation in the presence of LPS

Various cell concentrations of rat spleen lymphocytes were cultured with 10 ug/ml of LPS in the well of a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

3.1.3 Time Dependence of ^{3}H-Thymidine Incorporation

To examine whether ^{3}H-thymidine incorporation by rat spleen lymphocytes was different at different time points during the 72 hour incubation period, the cultures were pulse-labelled with ^{3}H-thymidine for 6 hours at different time points. As shown in Fig. 6, the basal ^{3}H-thymidine incorporation was very stable throughout the 72 hour incubation period except for an initial drop after the first 12 hours.

To study the optimal time point for the mitogens to exert their mitogenic effects on the rat spleen lymphocytes, similar pulse labelling experiments were repeated in the presence of con A and LPS. If one defines the mitogenic index as the ratio of ^{3}H-thymidine incorporation in the presence of mitogen to that in its absence, it is apparent from Fig. 7 and 8 that the optimal mitogenic effect for both con A and LPS can be obtained after 48 hours of mitogen treatment. Consequently, the spleen lymphocytes were pulsed with ^{3}H-thymidine at 48 hours and harvested at 54 hours in all subsequent experiments.

3.2 Effects of Adrenergic and Cholinergic Drugs on ^{3}H-Thymidine Incorporation

3.2.1 Action of Adrenergic Drugs and Carbachol on ^{3}H-Thymidine Incorporation

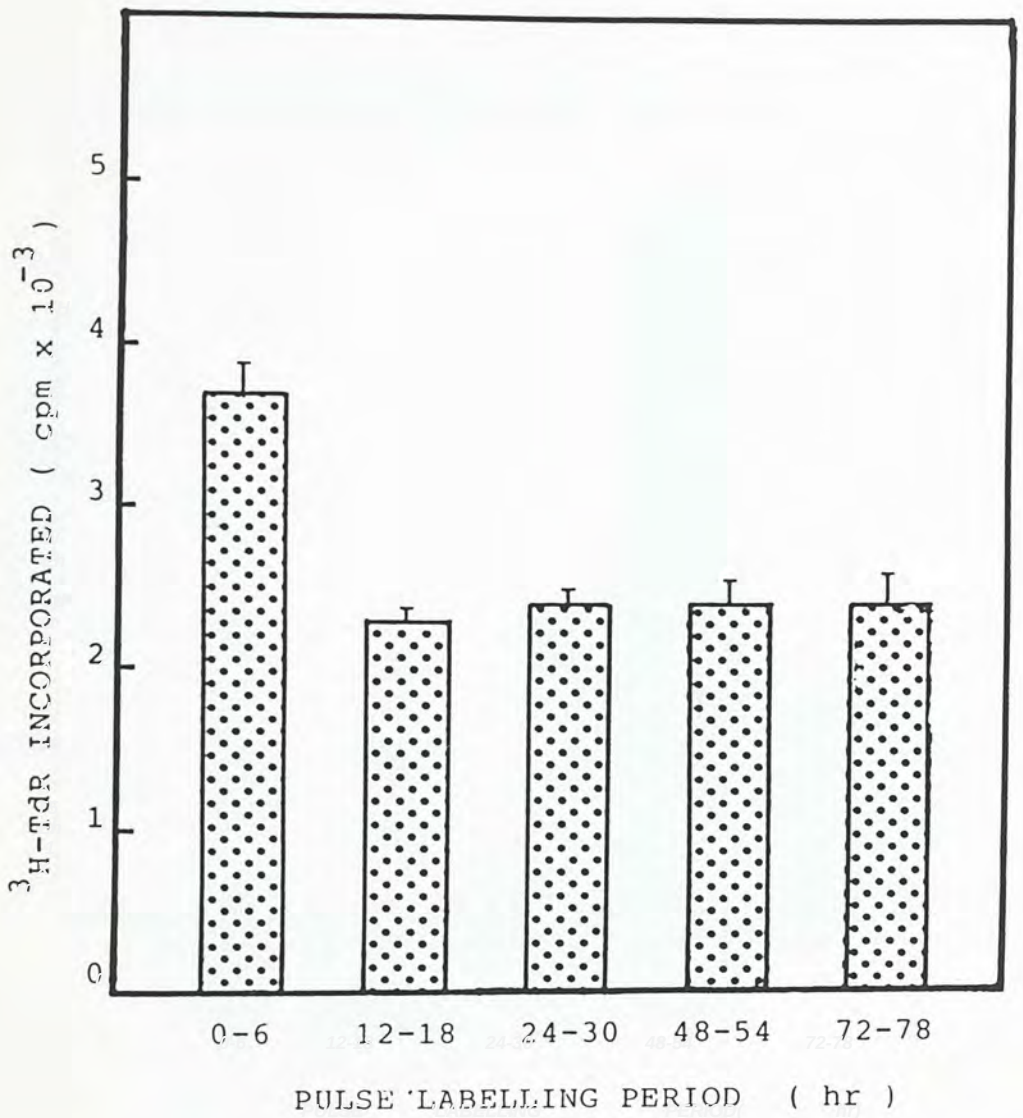

Fig. 6 Time dependence of ^{3}H-thymidine incorporation in the absence of mitogens

10^6 rat spleen lymphocytes were cultured in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 48 or 72 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. After which, the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

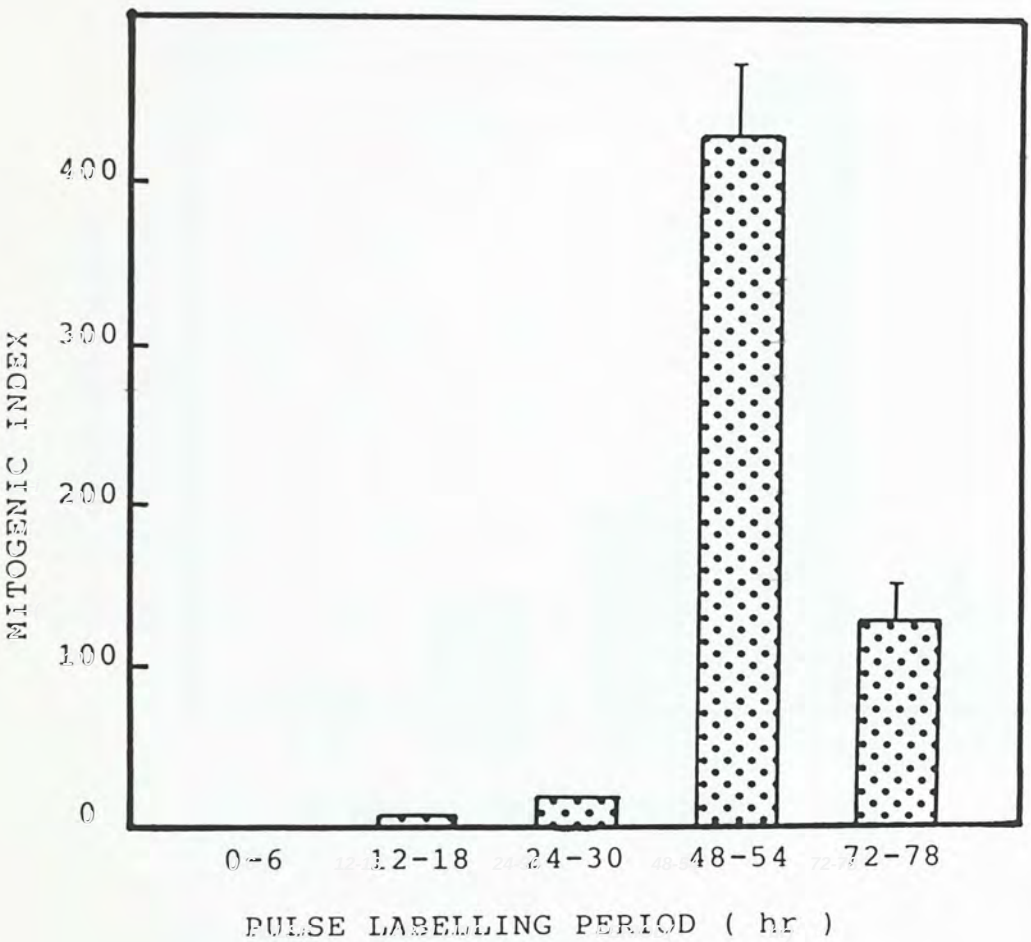

Fig. 7 Time dependence of ^{3}H-thymidine incorporation in the presence of con A

3 x 10^{5} rat spleen lymphocytes were cultured with 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 48 or 72 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. After which, the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments. Mitogenic index is the ratio of ^{3}H-thymidine incorporation in the presence of mitogen to that in its absence.

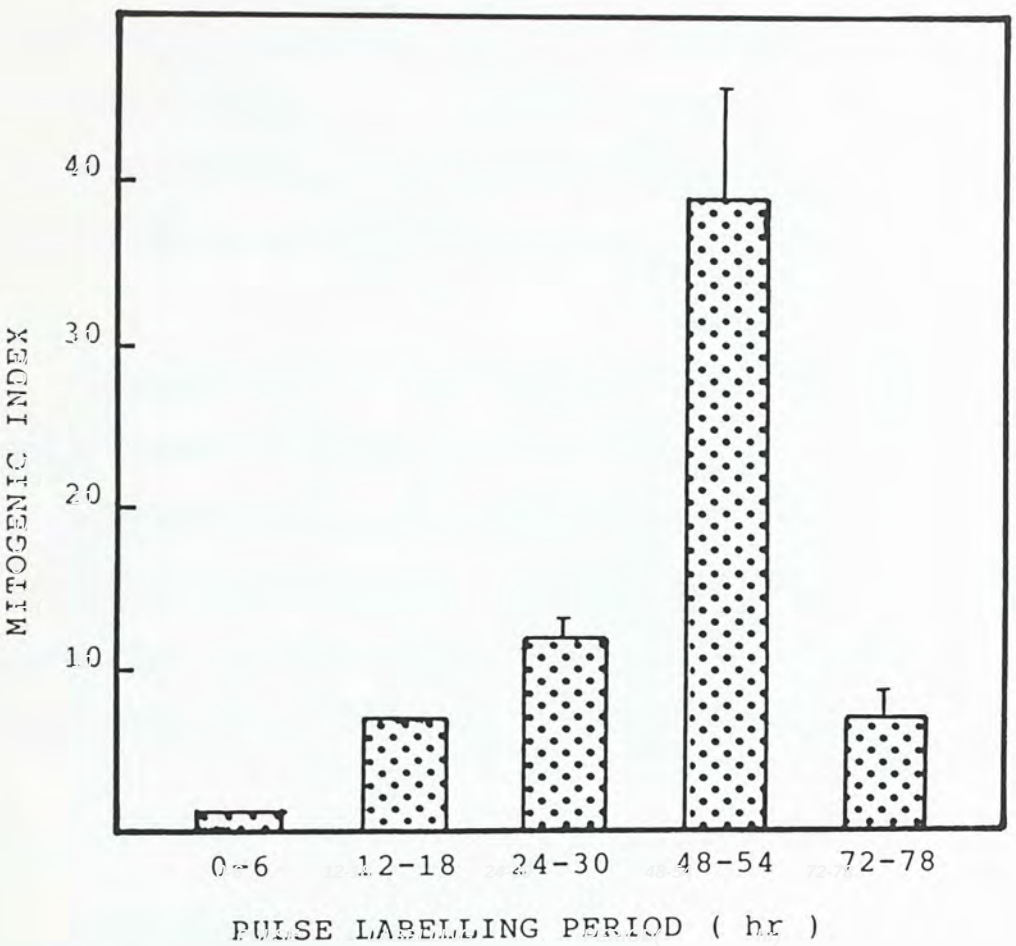

Fig. 8 Time dependence of ^{3}H-thymidine incorporation in the presence of LPS

3 X 10^{5} rat spleen lymphocytes were cultured with 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 48 or 72 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. After which, the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments. Mitogenic index is the ratio of ^{3}H-thymidine incorporation in the presence of mitogen to that in its absence.

All three adrenergic drugs tested, namely, isoproterenol, norepinephrine and epinephrine, showed a dose-dependent suppression of the basal (Fig. 9), con A-induced (Fig. 10) and LPS-induced (Fig. 11) ^{3}H-thymidine incorporation into the rat spleen lymphocytes. IC_{50} (concentration of drugs which gives 50 % inhibition) of the drugs were between 10 and 100 uM.

Carbachol did not seem to have any significant effect on the con A-induced ^{3}H-thymidine incorporation (Fig. 13) but exhibited a slight but significant inhibition of the basal incorporation at 100 uM and 1 mM on the basal incorporation (Fig. 12). Significant inhibition of LPS-induced ^{3}H-thymidine incorporation was also observed at 10 uM, 100 uM and 1 mM of carbachol (Fig. 14).

3.2.2 Cytotoxicity of Adrenergic Drugs

The viability of spleen lymphocytes in the presence of 100 uM isoproterenol, norepinephrine or epinephrine was not significantly different from that of the control. However, in the presence of 1 mM of these adrenergic drugs, the cell viability was significantly lower than that of the control (Table 1). In other words, 100 uM of these drugs were non-cytotoxic whereas they were toxic at 1 mM. Consequently, 100 uM of the adrenergic drugs was employed in all subsequent experiments in order to obtain a non-cytotoxic suppression of ^{3}H-thymidine incorporation.

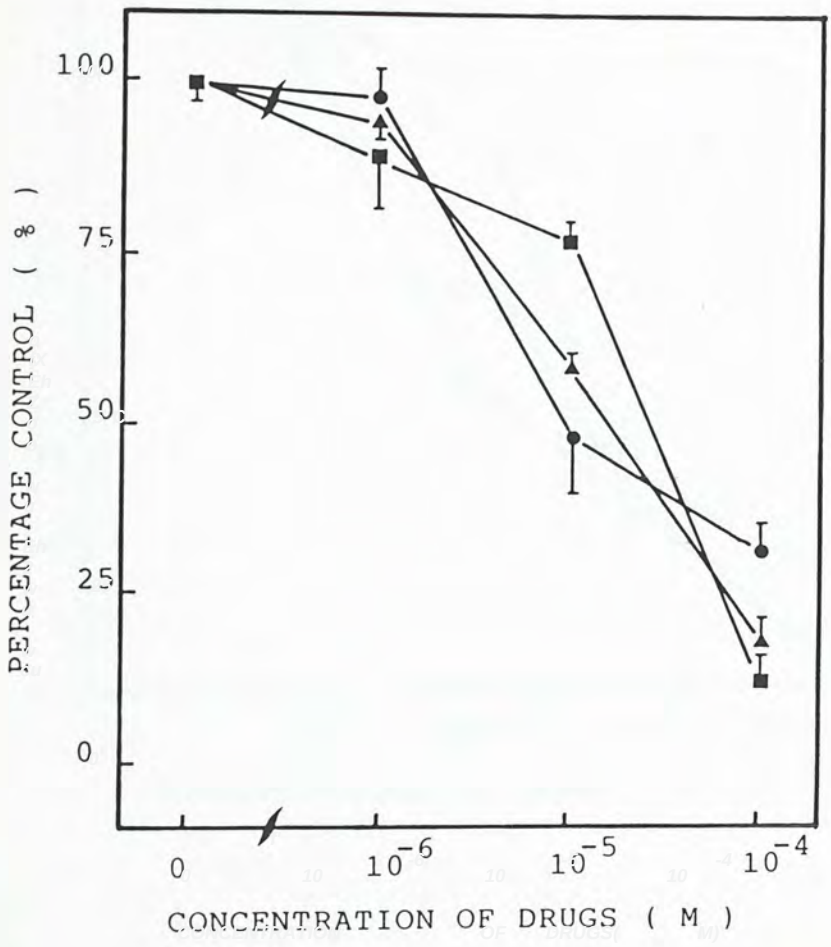

Fig. 9 Action of adrenergic drugs on the basal ^{3}H-thymidine incorporation

10^{6} rat spleen lymphocytes were cultured with different concentrations of isoproterenol (■), norepinephrine (▲) or epinephrine (●) in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 5,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

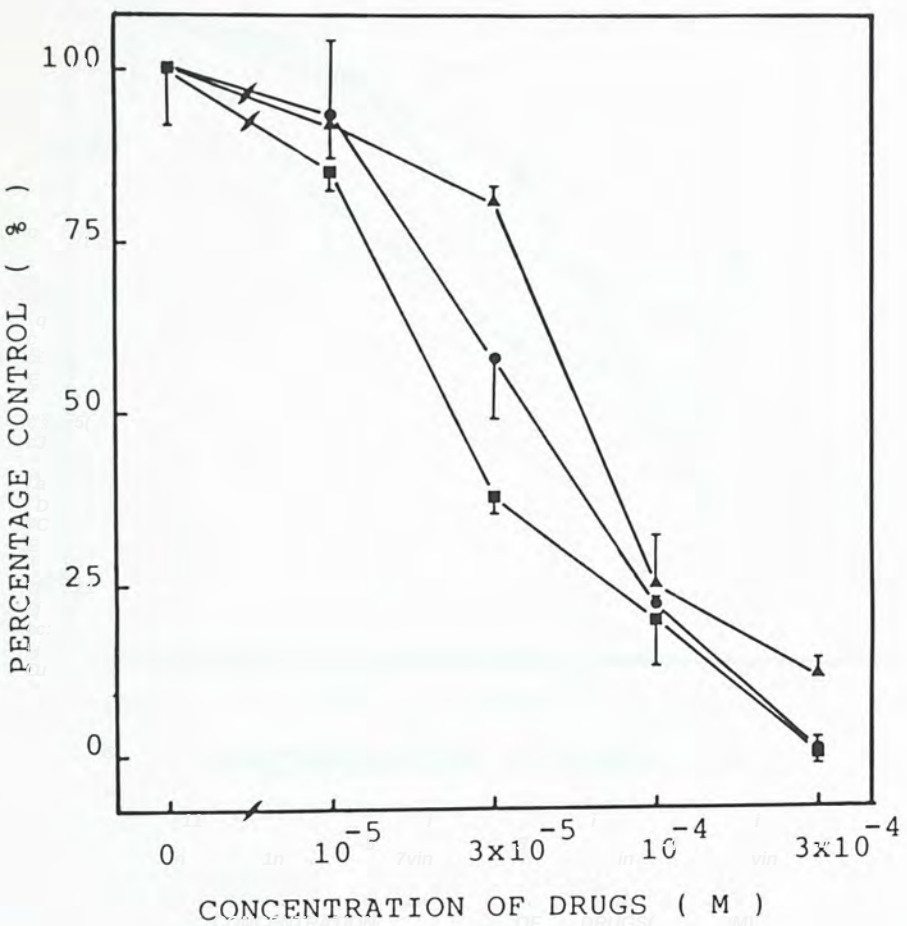

Fig. 10 Action of adrenergic drugs on the con A-induced ^{3}H-thymidine incorporation

3 x 10^5 rat spleen lymphocytes were cultured with different concentrations of isoproterenol (■), norepinephrine (▲) or epinephrine (●) in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence was 50,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

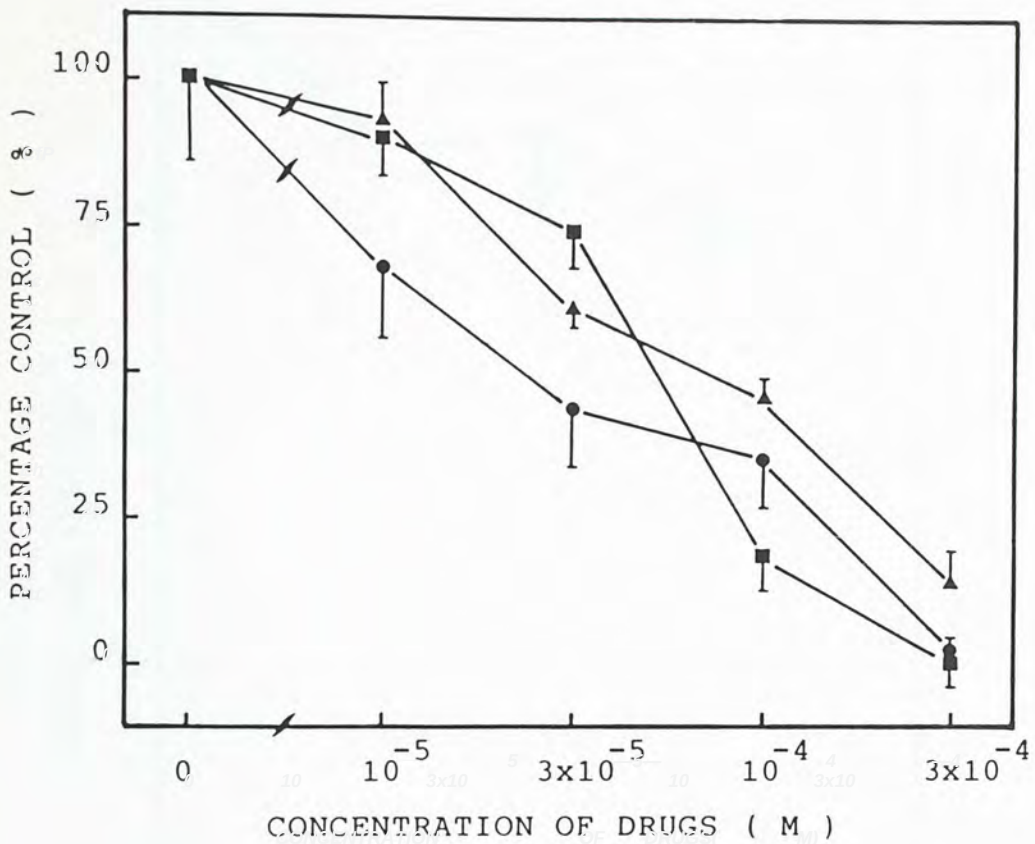

Fig. 11 Action of adrenergic drugs on the LPS-induced ^{3}H-thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were cultured with different concentrations of isoproterenol (■), norepinephrine (▲) or epinephrine (●) in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 10,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

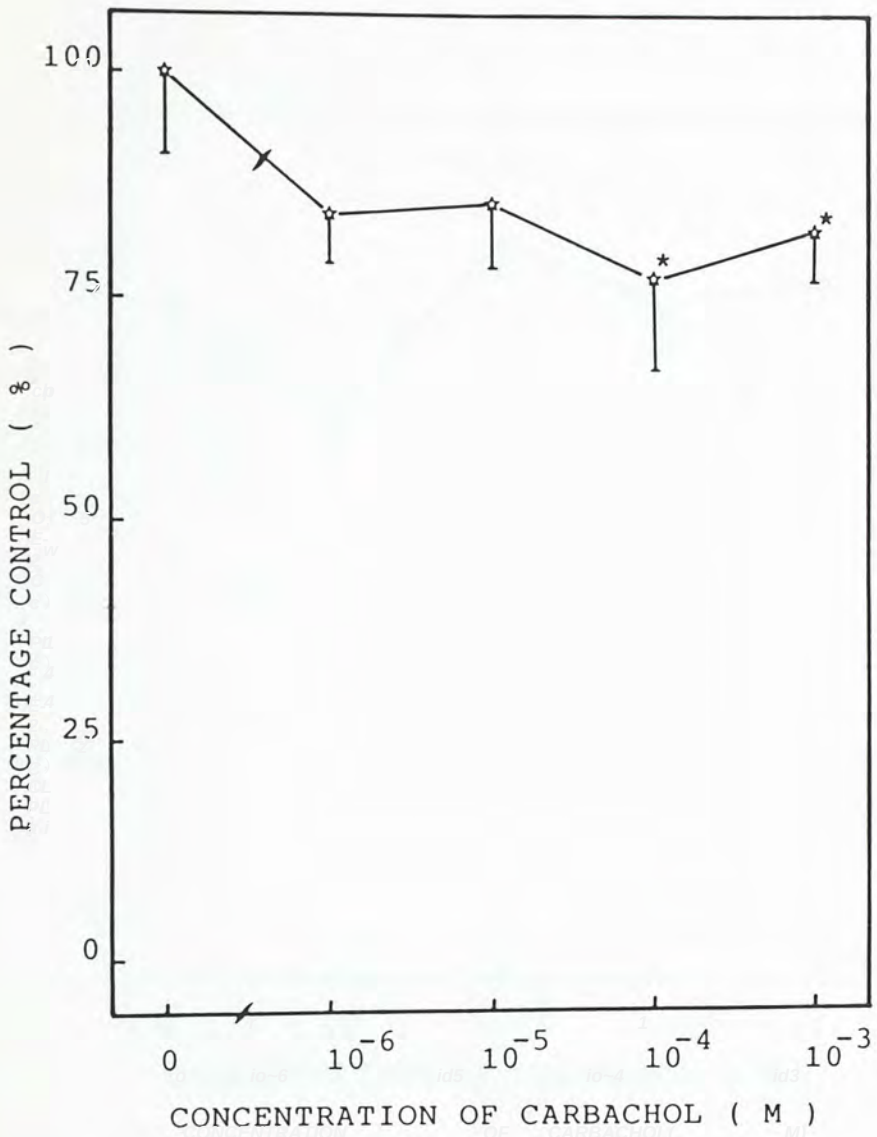

Fig. 12 Action of carbachol on the basal ^{3}H-thymidine incorporation

10^{6} rat spleen lymphocytes were cultured with different concentrations of carbachol in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 5,000 cpm. Data given are the means $\pm$ S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

*Significantly different from the control ($P<0.05$).

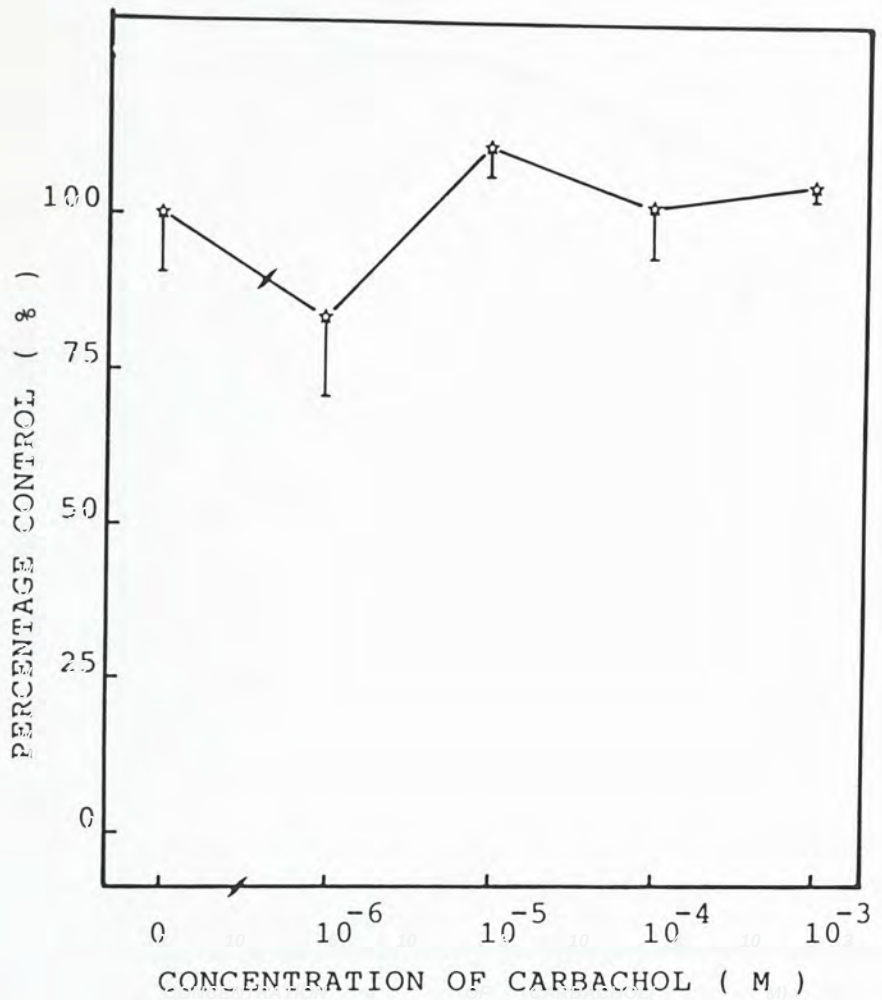

Fig. 13 Action of carbachol on the con A-induced ^{3}H-thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were cultured with different concentrations of carbachol in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 50,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

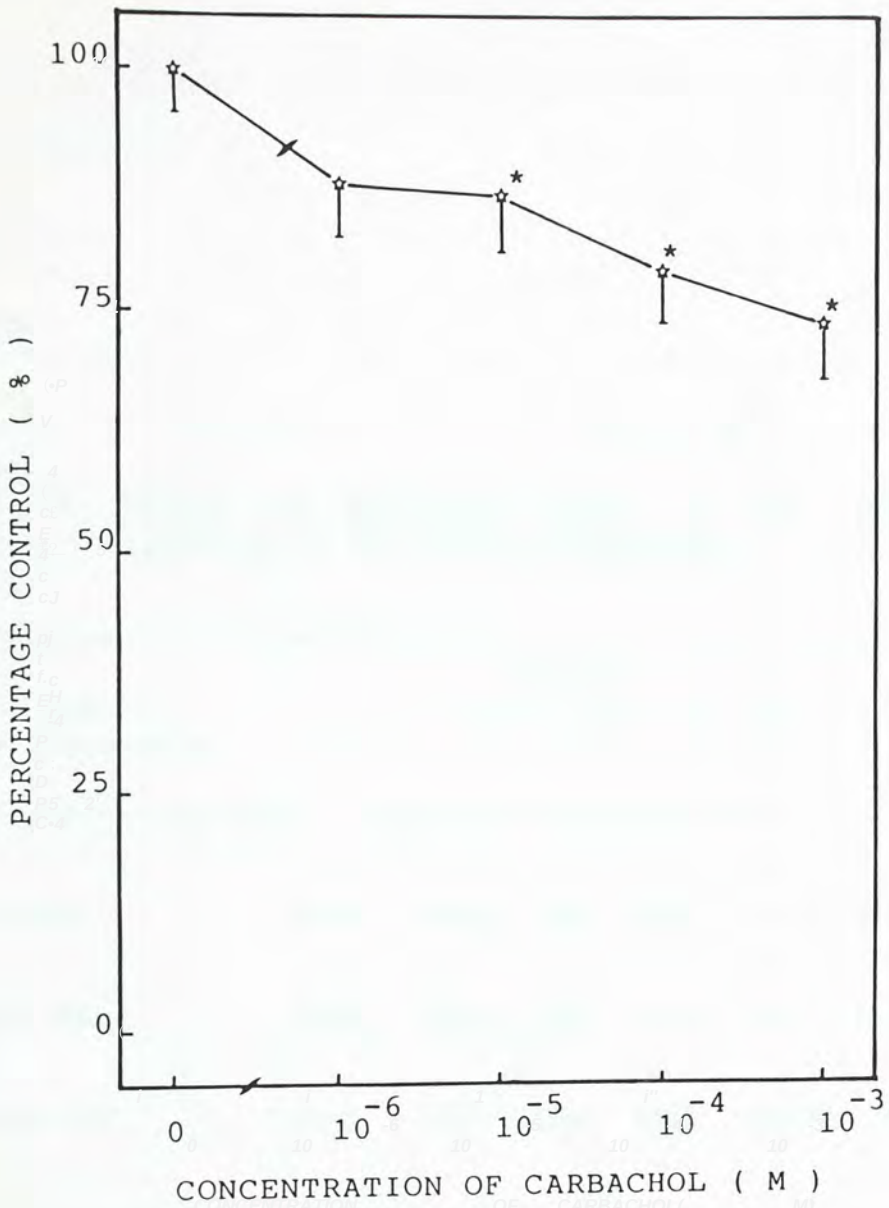

Fig. 14 Action of carbachol on the LPS-induced ^{3}H-thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were cultured with different concentrations of carbachol in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37 °C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 10,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

*Significantly different from the control ($P<0.05$).

Table 1 Effect of adrenergic drugs on the percentage viability of rat spleen lymphocytes

% viability in the presence of	C	100 uM[a]			1 mM[b]		
		I	N	E	I	N	E
no mitogen	61±6	60±2	58±2	62±1	37±5	37±1	49±1
1 ug/ml con A	49±1	43±11	49±5	47±6	28±5	15±3	26±3
10 ug/ml LPS	44±8	46±1	49±4	43±5	29±3	21±1	22±8

Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in two separate experiments.

[a]Not significantly different from control ($P>0.05$).

[b]Significantly lower than control ($P<0.05$).

Abbreviations: C, control; I, isoproterenol; N, norepinephrine; E, epinephrine.

3.2.3 Time Dependence of Adrenergic Drug Action on ^{3}H-Thymidine Incorporation

The suppressive action of isoproterenol, norepinephrine or epinephrine on the basal,con A-induced and LPS-induced ^{3}H-thymidine incorporation was most potent when the drugs were added at the beginning of the incubation period, the suppressive effects were progressively lower when the drugs were added at later time points (Fig. 15-17).

3.3 Effects of cAMP on ^{3}H-Thymidine Incorporation

3.3.1 Effects of cAMP Elevating Drugs on ^{3}H-Thymidine Incorporation

Dibutyryl cAMP is a cAMP derivative. Theophylline and IBMX are cAMP phosphodiesterase inhibitors. Addition of these drugs into the lymphocyte cultures will mimic the action of or lead to an increase in cAMP level in the lymphocytes. All three drugs showed a dose-dependent suppression of the basal, con A-induced and LPS-induced ^{3}H-thymidine incorporation suggesting that cAMP was inhibitory to lymphocyte proliferation (Fig. 18-20).

3.3.2 Cytotoxicity of the cAMP Elevating Drugs

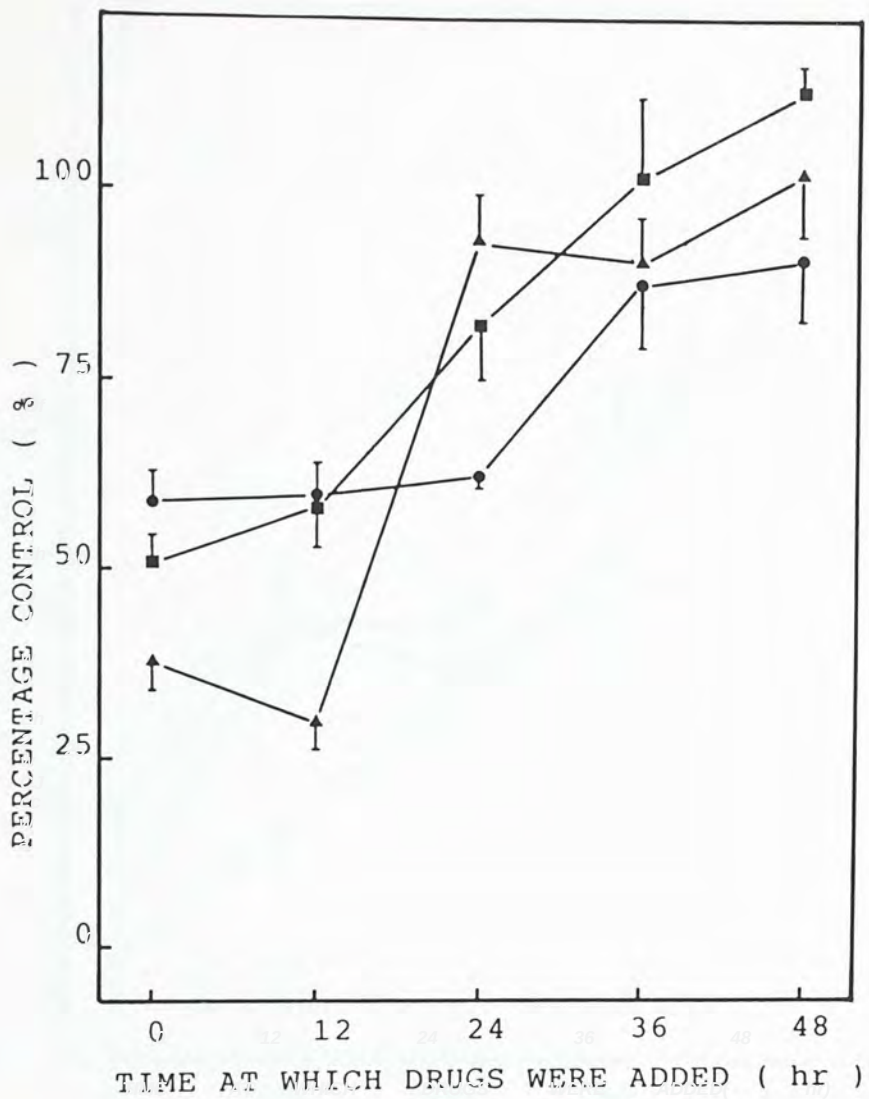

Fig. 15 Time dependence of adrenergic drug action on the basal ^{3}H-thymidine incorporation

10^{6} rat spleen lymphocytes were exposed to 100 uM isoproterenol (■), norepinephrine (▲) or epinephrine (●) in a flat-bottomed 96 well microtiter plate after 0, 12, 24, 36 or 48 hrs of incubation at 37°C. At 48 hrs of incubation, 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 5,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

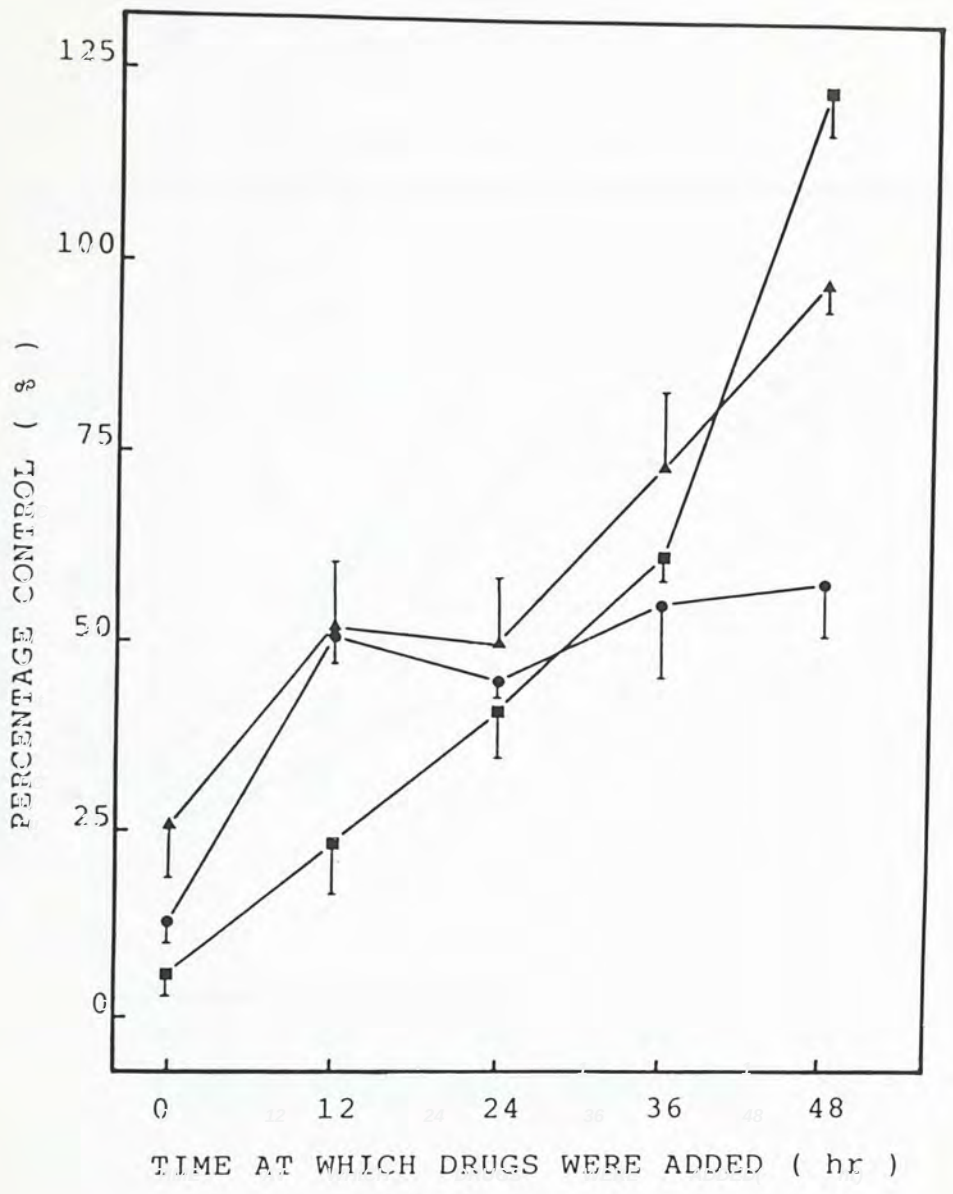

Fig. 16 Time dependence of adrenergic drug action on the con A-induced ^{3}H -thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were exposed to 100 uM isoproterenol (■), norepinephrine (▲) or epinephrine (●) in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate after 0, 12, 24, 36 or 48 hrs of incubation at 37°C. At 48 hrs of incubation, 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 50,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

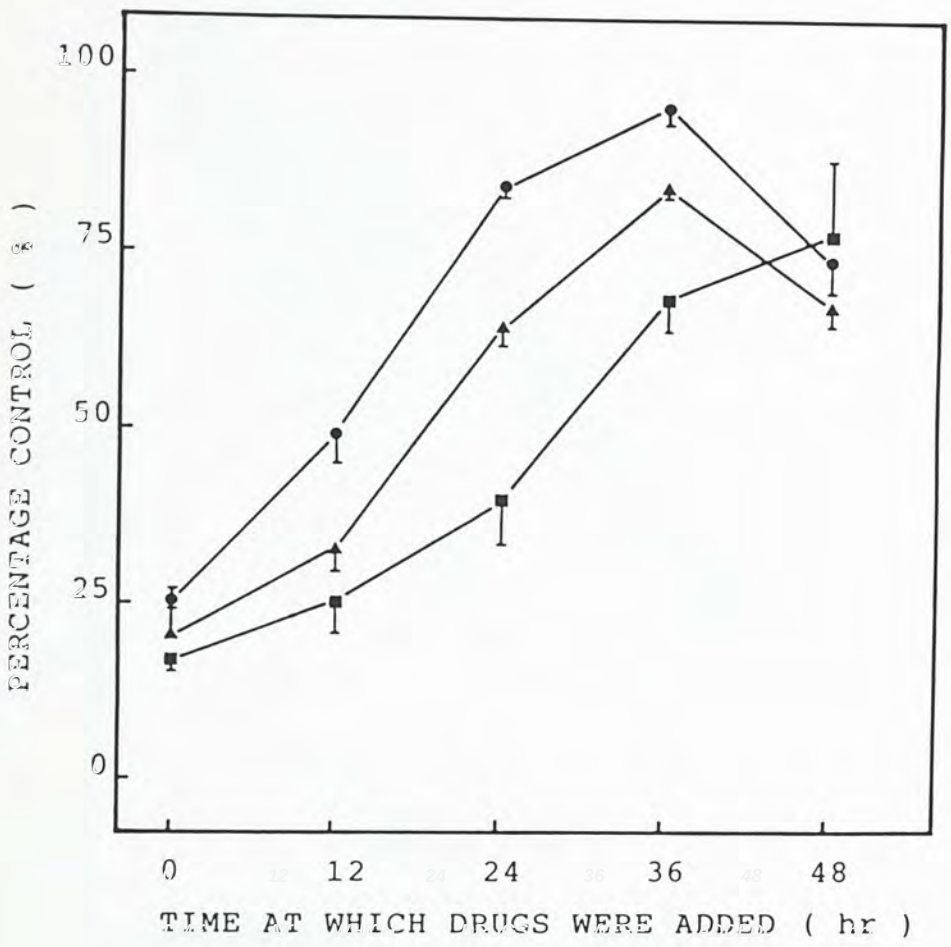

Fig. 17 Time dependence of adrenergic drug action on the LPS-induced ^{3}H -thymidine incorporation

3 x 10^5 rat spleen lymphocytes were exposed to 100 uM isoproterenol (■), norepinephrine (▲) or epinephrine (●) in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate after 0, 12, 24, 36 or 48 hrs of incubation at 37°C. At 48 hrs of incubation, 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 10,000 cpm. Data given are the means $\pm$ S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

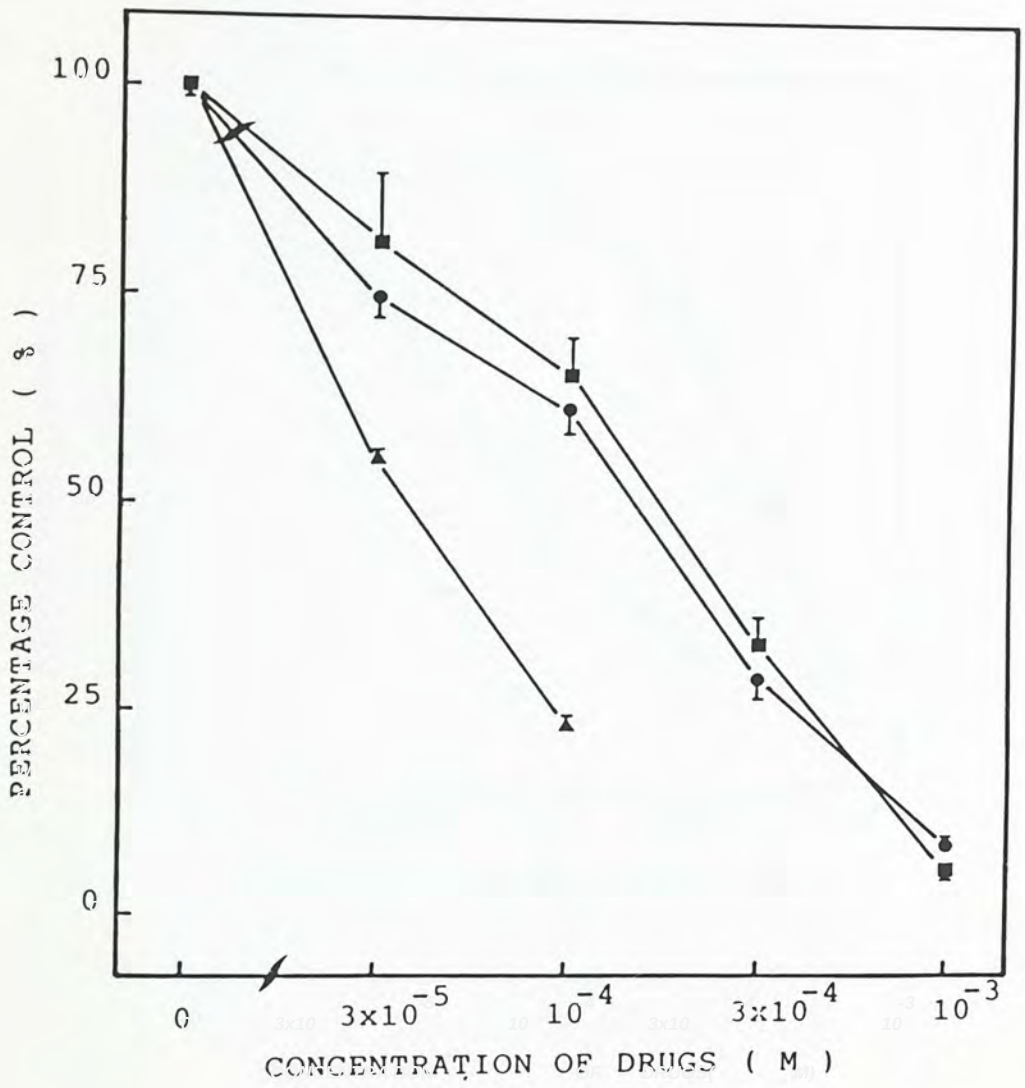

Fig. 18 Effect of cAMP elevating drugs on the basal ^{3}H-thymidine incorporation

10^{6} rat spleen lymphocytes were cultured with different concentrations of dibutyryl cAMP (●), theophylline (■) or IBMX (▲) in a flat-bottomed 96 well microtiter plate for 48 hrs at 37 °C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 5,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

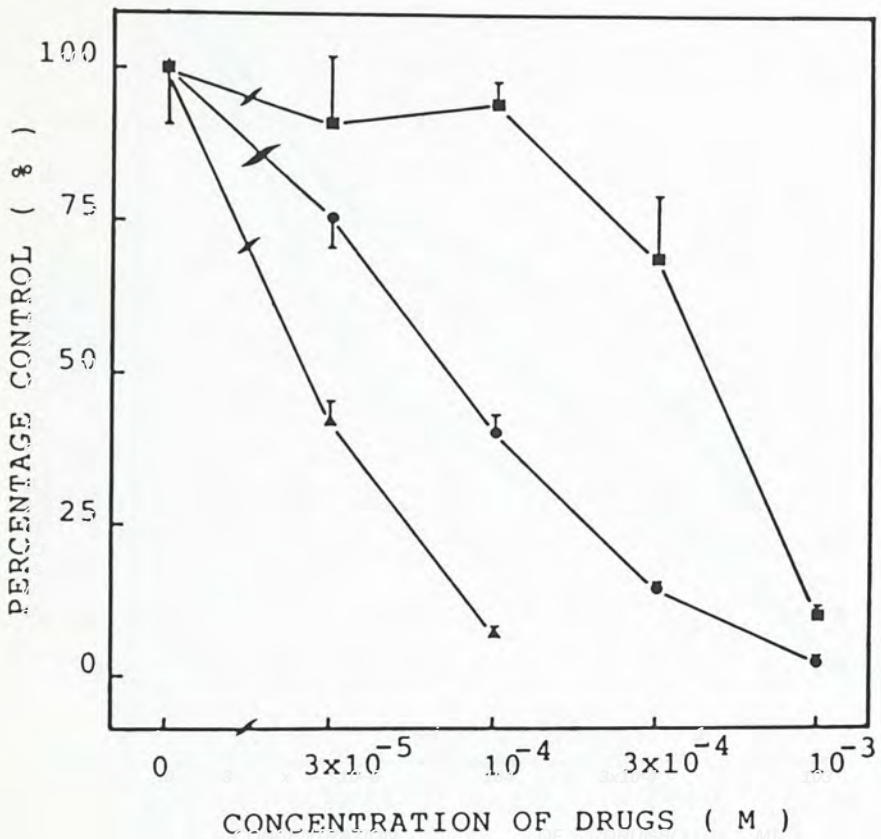

Fig. 19 Effect of cAMP elevating drugs on the con A-induced ^{3}H-thymidine incorporation

3 x 10^5 rat spleen lymphocytes were cultured with different concentrations of dibutyryl cAMP (●), theophylline (■) or IBMX (▲) in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37 °C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 50,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

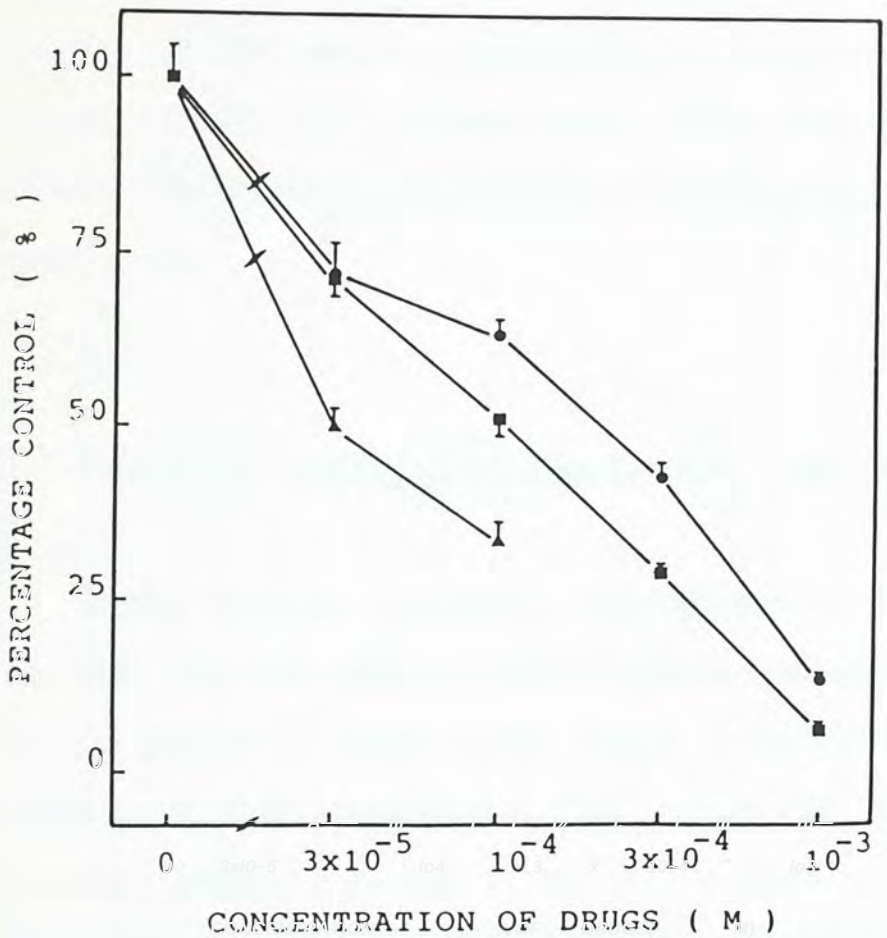

Fig. 20 Effect of cAMP elevating drugs on the LPS-induced ^{3}H-thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were cultured with different concentrations of dibutyryl cAMP (●), theophylline (■) or IBMX (▲) in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37 °C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The typical amount of ^{3}H-thymidine incorporated in the absence of drugs was 10,000 cpm. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

The viability of spleen lymphocytes in the presence of 300 uM and 1 mM of dibutyryl cAMP or theophylline as well as 30 uM and 100 uM IBMX was not significantly different from that of the control (Table 2). In other words, these drugs were able to suppress ^{3}H-thymidine incorporation without actually killing the lymphocytes.

3.3.3 Cyclic AMP Accumulation stimulated by Isoproterenol

Within 2 min of incubation, isoproterenol (1 uM) was able to increase the cAMP level in the rat spleen lymphocytes from less than 1 pmoles/10^6 cells to more than 6 pmoles/10^6 cells in the presence of 10 mM theophylline. This rise in cAMP level reached a plateau between 2 and 3 min of incubation (Fig. 21). Isoproterenol elicited a dose-dependent increase in cAMP level (Fig. 22). The maximal response occurred at about 1 uM isoproterenol.

3.3.4 Time Dependence of cAMP Elevating Drugs on ^{3}H-Thymidine Incorporation

As shown in Fig. 23 to 25, the inhibitory action of dibutyryl cAMP, theophylline and IBMX on the basal, the con A-induced and the LPS-induced ^{3}H-thymidine incorporation was most potent when the drugs were added at the beginning of the

Table 2 Effect of dibutyryl cAMP, theophylline and IBMX on the percentage viability of rat spleen lymphocytes

% viability in the presence of	C	D		T		X	
		100uM	1mM	100uM	1mM	30uM	100uM
no mitogen	50±3	50±4	54±5	50±6	55±7	55±7	53±3
1 ug/ml con A	30±4	28±6	28±4	31±4	30±6	29±7	24±3
10 ug/ml LPS	25±5	22±1	29±1	26±3	26±6	21±5	23±6

Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in two separate experiments.

All test groups were not significantly different from control ($P>0.05$).

Abbreviations: C, control; D, dibutyryl cAMP; T, theophylline; X, IBMX.

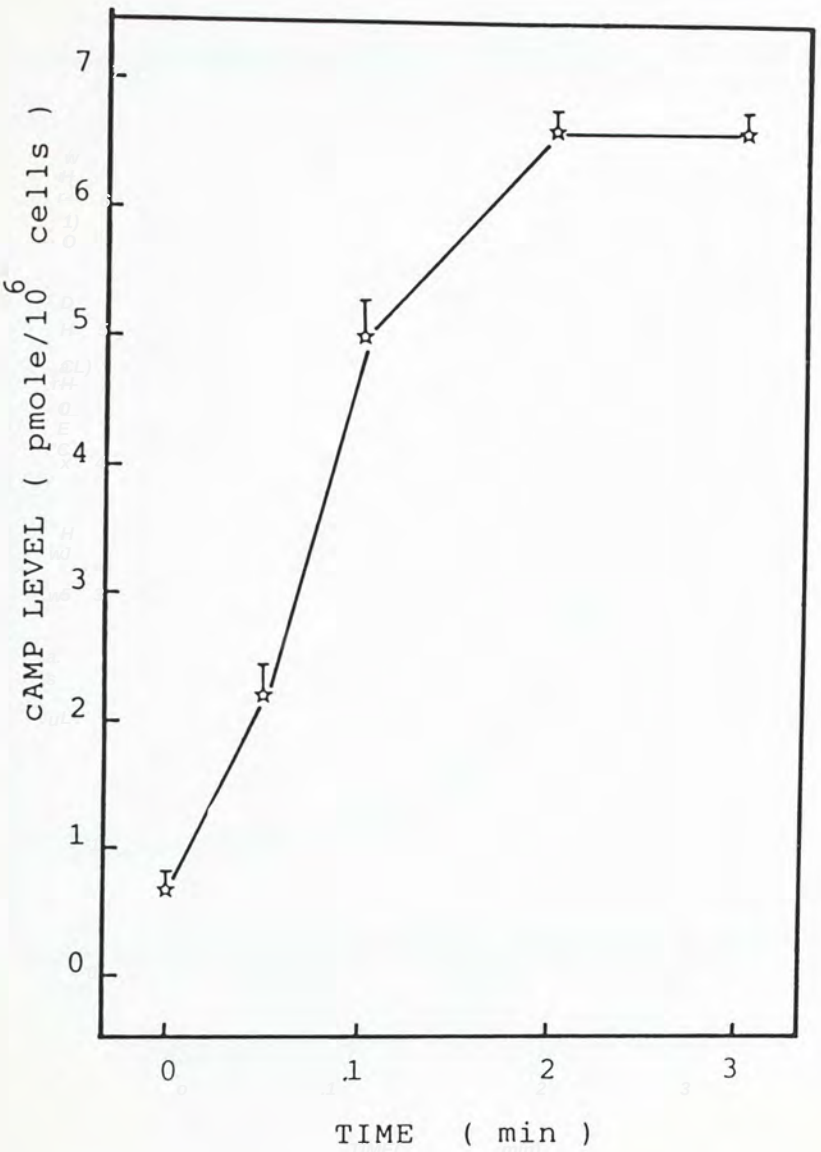

Fig. 21 Time dependence of cAMP accumulation by isoproterenol stimulation

Rat spleen lymphocytes were incubated in the presence of 1 uM isoproterenol and 10 mM theophylline at 37°C for various time periods. The reaction mixture was then boiled for 4 min to stop the reaction. Precipitates formed were centrifuged at 10,000 x g for 15 min at room temperature. 50 ul of the supernatant was used for cAMP determination. Data given are the means ± S.D. of duplicate determinations. Similar results were obtained in 2 separate experiments.

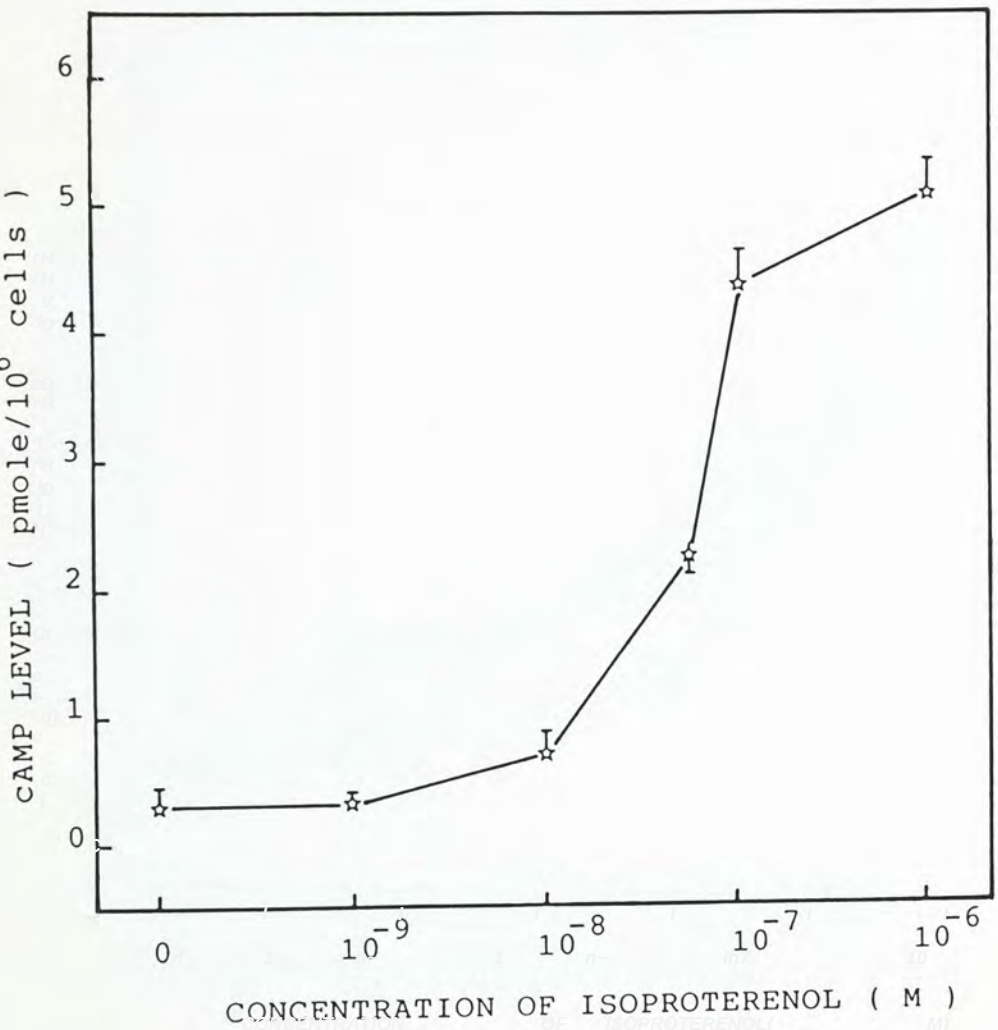

Fig. 22 Dose response curve of isoproterenol on cAMP accumulation

Rat spleen lymphocytes were incubated at 37°C for 1 min in the presence of different concentrations of isoproterenol and 10 mM theophylline. The reaction mixture was then boiled for 4 min to stop the reaction. Precipitates formed were centrifuged at 10,000 x g for 15 min at room temperature. 50 ul of the supernatant was used for cAMP determination. Data given are the means ± S.D. of duplicate determinations. Similar results were obtained in 2 separate experiments.

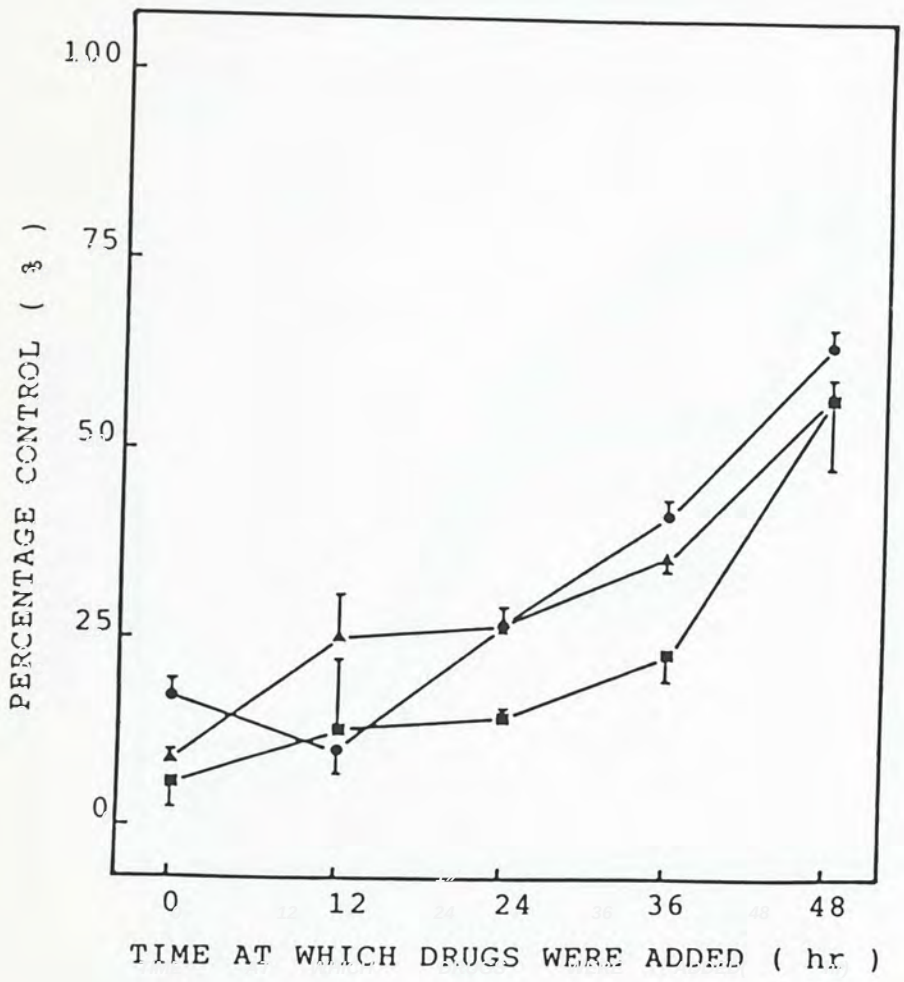

Fig. 23 Time dependence of cAMP elevating durgs on the basal ^{3}H-thymidine incorporation

10^{6} rat spleen lymphocytes were cultured with 100 uM IBMX (▲), 1 mM theophylline (■) or 1 mM dibutyryl cAMP (●) in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 36 or 48 hrs at 37 °C. At 48 hrs of incubation 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

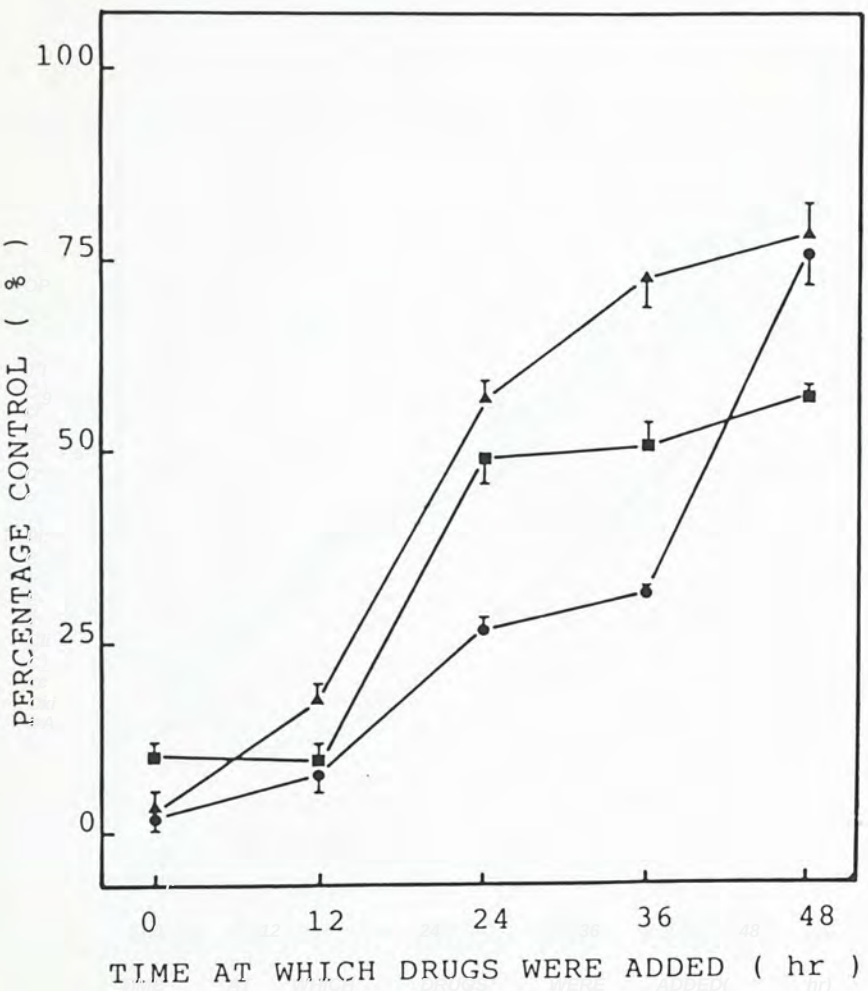

Fig. 24 Time dependence of cAMP elevating drugs on the con A-induced ^{3}H-thymidine incorporation

3 x 10^{5} rat spleen lymphocytes were cultured with 100 uM IBMX (▲), 1 mM theophylline (■) or 1 mM dibutyryl cAMP (●) in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 36 or 48 hrs at 37 °C. At 48 hrs of incubation, 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

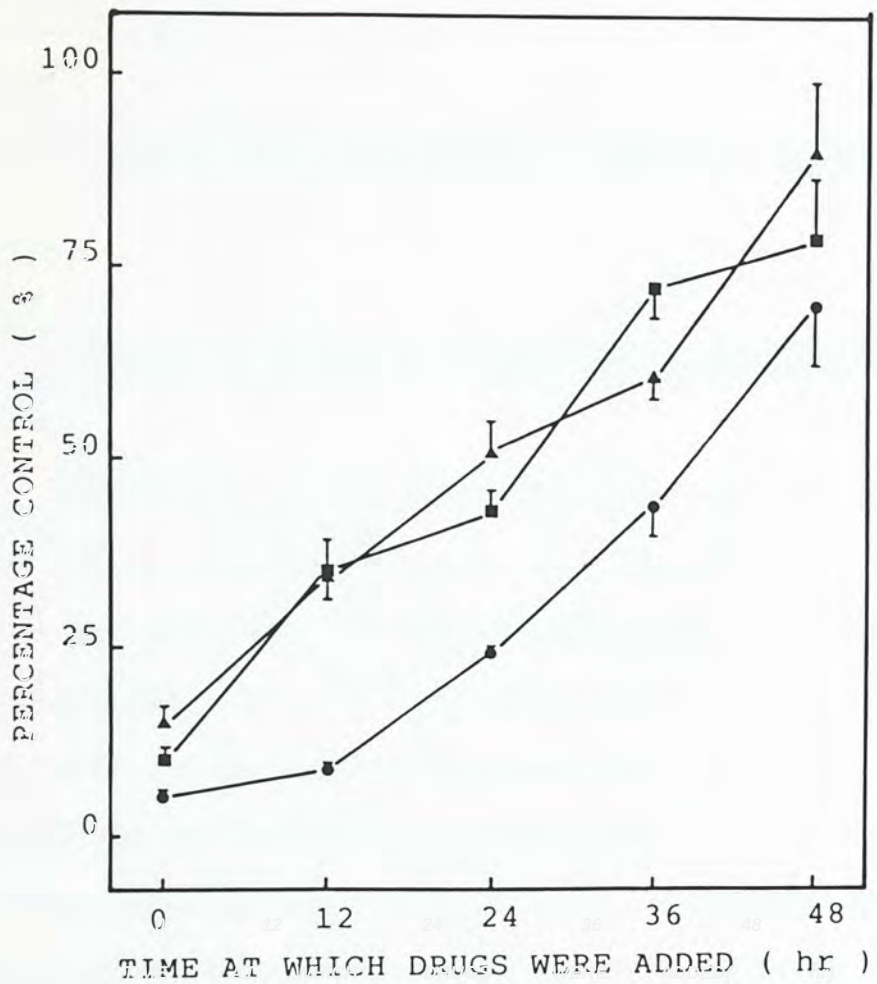

Fig. 25 Time dependence of cAMP elevating drugs on the LPS-induced ^{3}H-thymidine incorporation

3 x 10^5 rat spleen lymphocytes were cultured with 100 uM IBMX (▲), 1 mM theophylline (■) or 1 mM dibutyryl cAMP (●) in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 0, 12, 24, 36 or 48 hrs at 37 °C. At 48 hrs of incubation, 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

incubation period. The inhibition became less and less as the drugs were added at later time points.

3.4 Effects of A23187 and PMA on ^{3}H-Thymidine Incorporation

3.4.1 Effects of A23187 on ^{3}H-Thymidine Incorporation

A23187 is a calcium ionophore which provides ion channels on the plasma membrane and allows extracellular calcium ion to get into the cell down its concentration gradient. At a lower cell concentration (3×10^{5} cells per well), A23187 had a biphasic effect on the ^{3}H-thymidine incorporation (Fig. 26). It optimally stimulated the ^{3}H-thymidine incorporation at 1 uM. However, when the concentration was raised to 3 uM, it actually inhibited the basal incorporation of the label. A similar biphasic dose-response pattern was observed at a higher cell concentration (10^{6} cells per well). However, the optimal stimulation occurred at 300 nM, and A23187 became inhibitory at 1 uM.

3.4.2 Effects of PMA on ^{3}H-Thymidine Incorporation

Phorbol 12-myristate 13-acetate (PMA) is an activator of protein kinase C. It stimulated the basal ^{3}H-thymidine incorporation into the rat spleen lymphocytes in a dose-

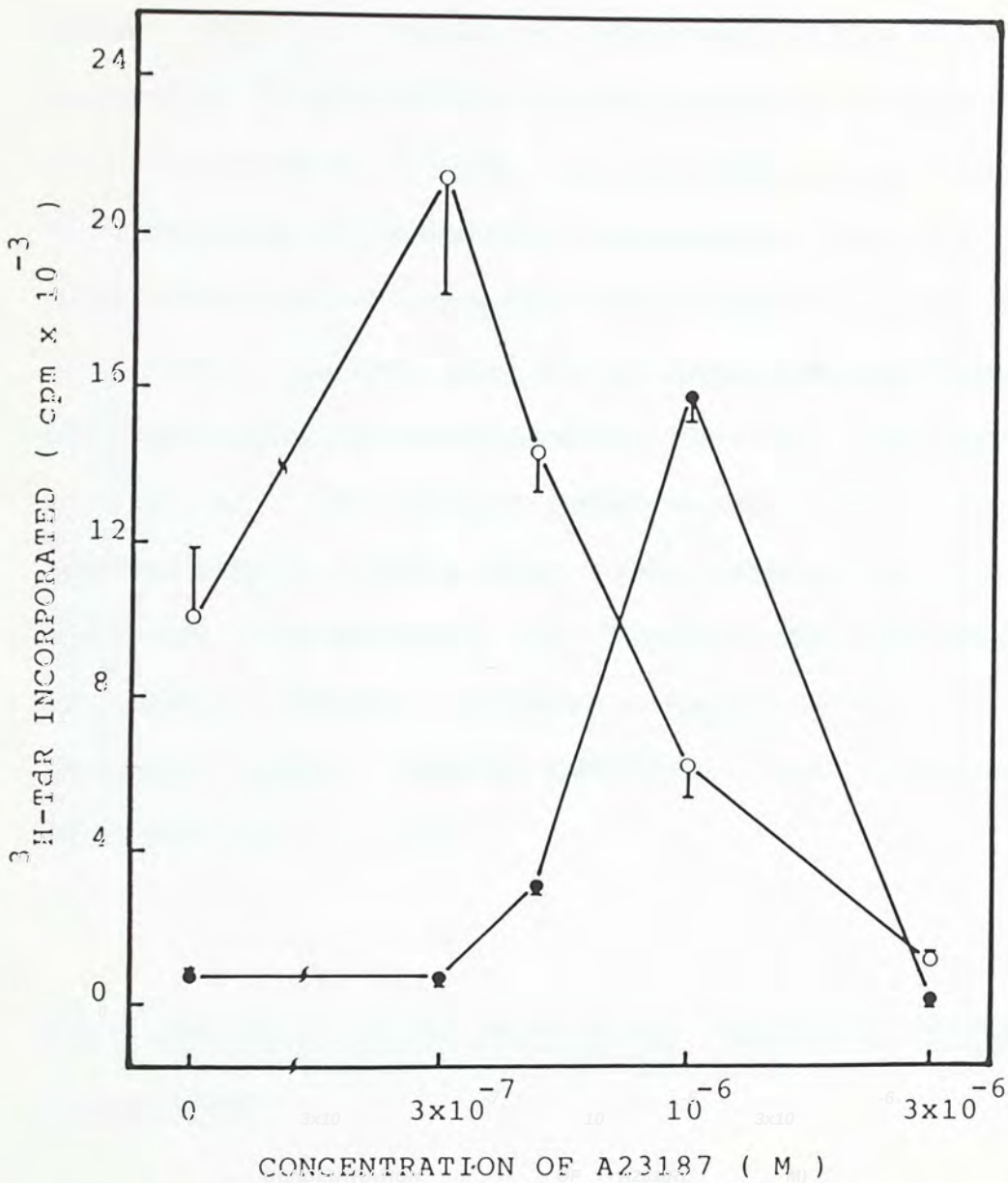

Fig. 26 Effect of A23187 on the basal ^{3}H-thymidine incorporation

3×10^5 (●) or 10^6 (O) rat spleen lymphocytes were cultured with different concentrations of A23187 in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

dependent manner (Fig. 27). In the presence of 1 ug/ml con A, however, PMA was inhibitory (Fig. 28). Complete inhibition occurred at 10 ug/ml of PMA. In the presence of 10 ug/ml LPS, PMA was stimulatory at 10 ng/ml. In a typical experiment (Table 3), the stimulation of ^{3}H-thymidine incorporation produced by LPS (10 ug/ml) alone and PMA (10 ng/ml) alone were 6615 cpm and 1476 cpm respectively. However, when the two drugs were added together to the lymphocytes, the stimulation was 19417 cpm. There was thus an increase of 11326 cpm which cannot be accounted for by simple addition effects of the 2 drugs. This indicates that LPS and PMA could act synergistically to stimulate DNA synthesis. The stimulation, however, decreased as the concentration of PMA was increased further. Complete inhibition, again, occurred at 10 ug/ml (Fig. 29).

3.4.3 Effects of Various Drugs on the PMA-Induced ^{3}H-Thymidine Incorporation

The presence of 100 uM of isoproterenol, norepinephrine, epinephrine, dibutyryl cAMP, IBMX and 1mM of theophylline were able to inhibit the PMA-induced ^{3}H-thymidine incorporation (Fig. 30). They were also inhibitory on the combined action of LPS (10 ug/ml) and PMA (10 ng/ml) (Fig. 32). However, when PMA was added together with con A in the lymphocyte culture, only the last three drugs were able to inhibit further the diminished response. The adrenergic drugs seemed to have lost their ability in suppressing ^{3}H-thymidine incorporation under these conditions

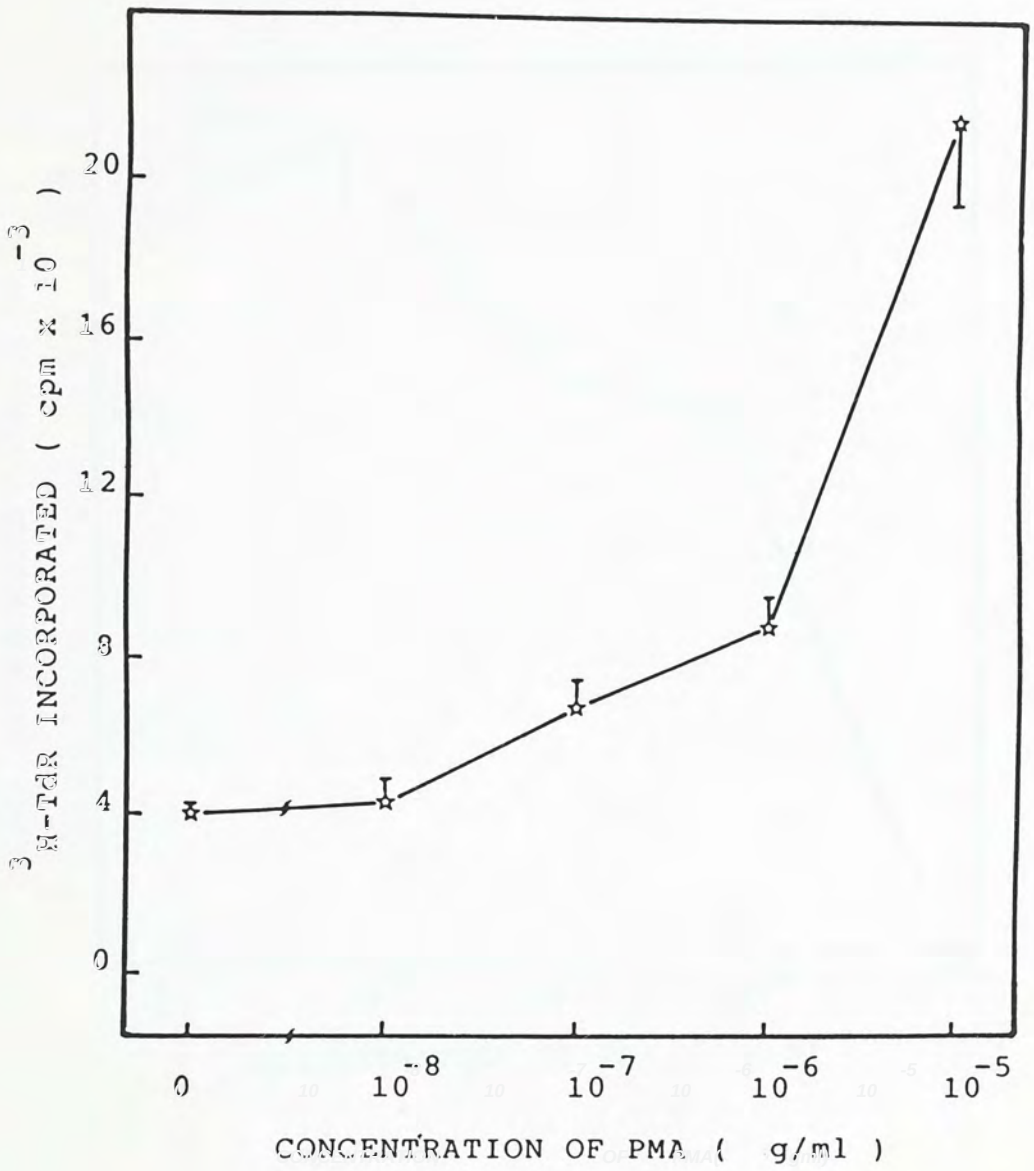

Fig. 27 Effect of PMA on the basal ^{3}H-thymidine incorporation

10^6 rat spleen lymphocytes were cultured with different concentrations of PMA in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

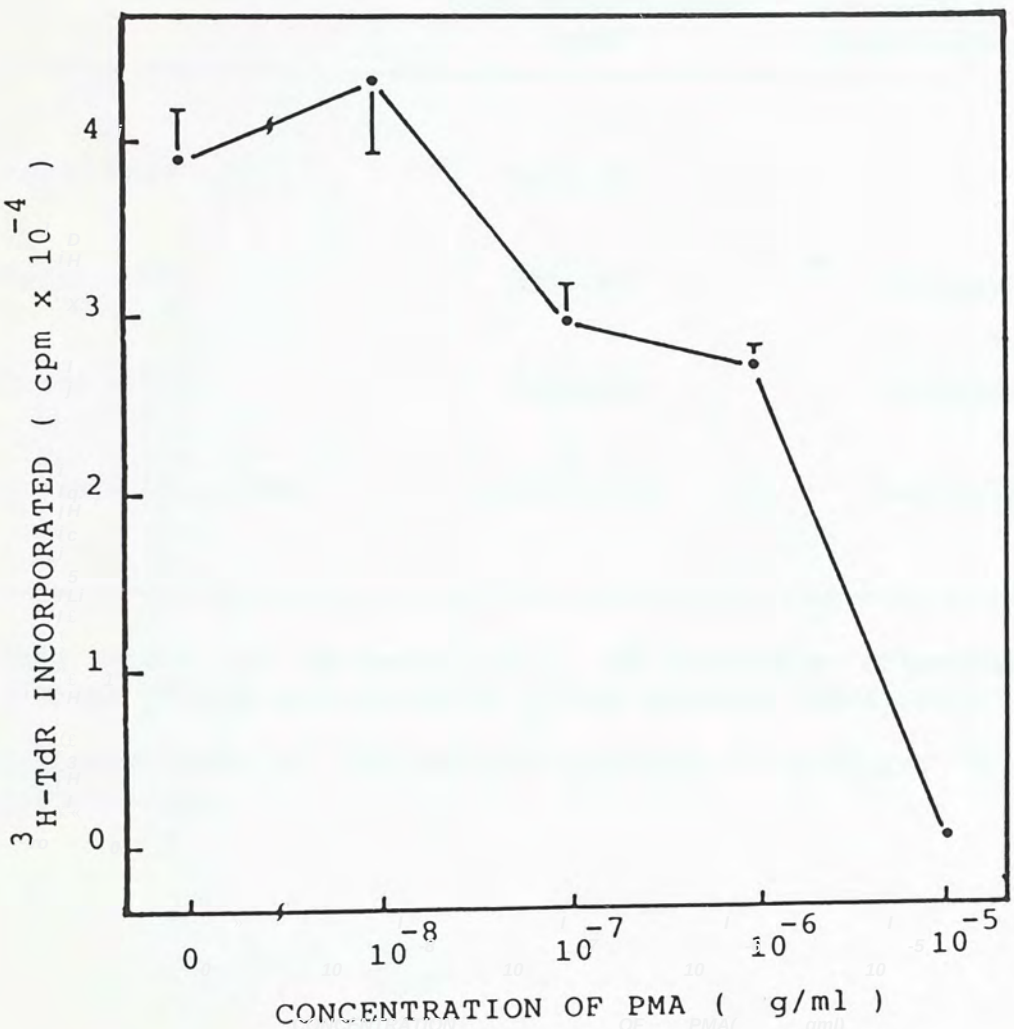

Fig. 28 Effect of PMA on the con A-induced ^{3}H-thymidine incorporation

3×10^5 rat spleen lymphocytes were cultured with different concentrations of PMA in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

Table 3 Synergism between LPS and PMA

	^{3}H-TdR incorporated (cpm)	increase in ^{3}H-TdR incorporated (cpm)
cells only	1419±193	-
cells + LPS	8034±417	6615±459
cells + PMA	2895±287	1476±346
cells + LPS + PMA	20836±1855	19417±1865

Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in two separate experiments.

Concentrations of LPS and PMA used were 10 ug/ml and 10 ng/ml respectively.

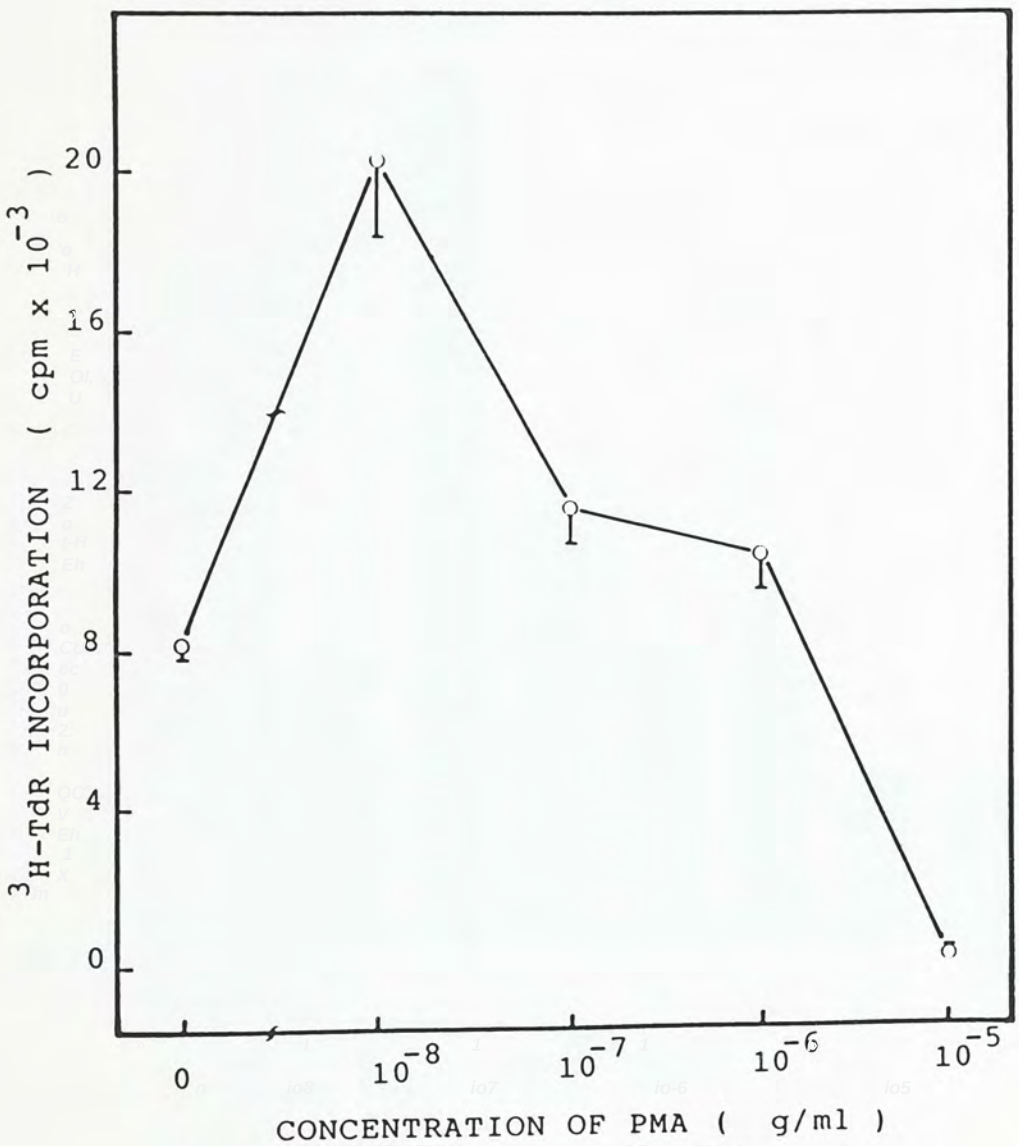

Fig. 29 Effect of PMA on the LPS-induced ^{3}H-thymidine incorporation

3×10^5 rat spleen lymphocytes were cultured with different concentrations of PMA in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. Similar results were obtained in 2 separate experiments.

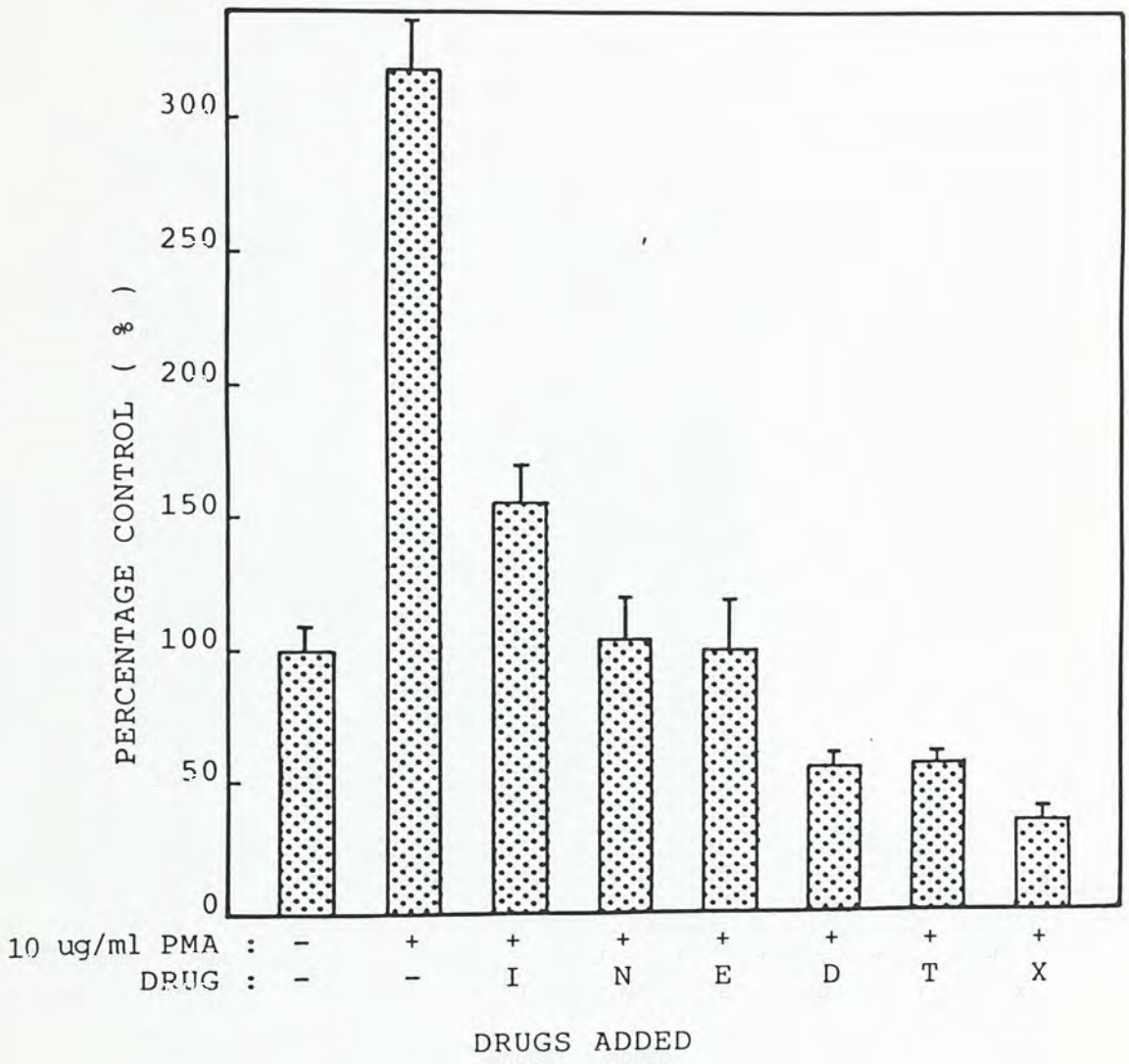

Fig. 30 Effect of various drugs on the basal ^{3}H-thymidine incorporation in the presence of PMA

10^6 rat spleen lymphocytes were cultured with various drugs as indicated above in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. The 100 % control corresponded to 5,000 cpm in a typical experiment. Similar results were obtained in 2 separate experiments.
Abbreviations: I, 100 uM isoproterenol; N, 100 uM norepinephrine; E, 100 uM epinephrine; D, 100 uM dibutyryl cAMP; T, 1 mM theophylline; X, 100 uM IBMX.

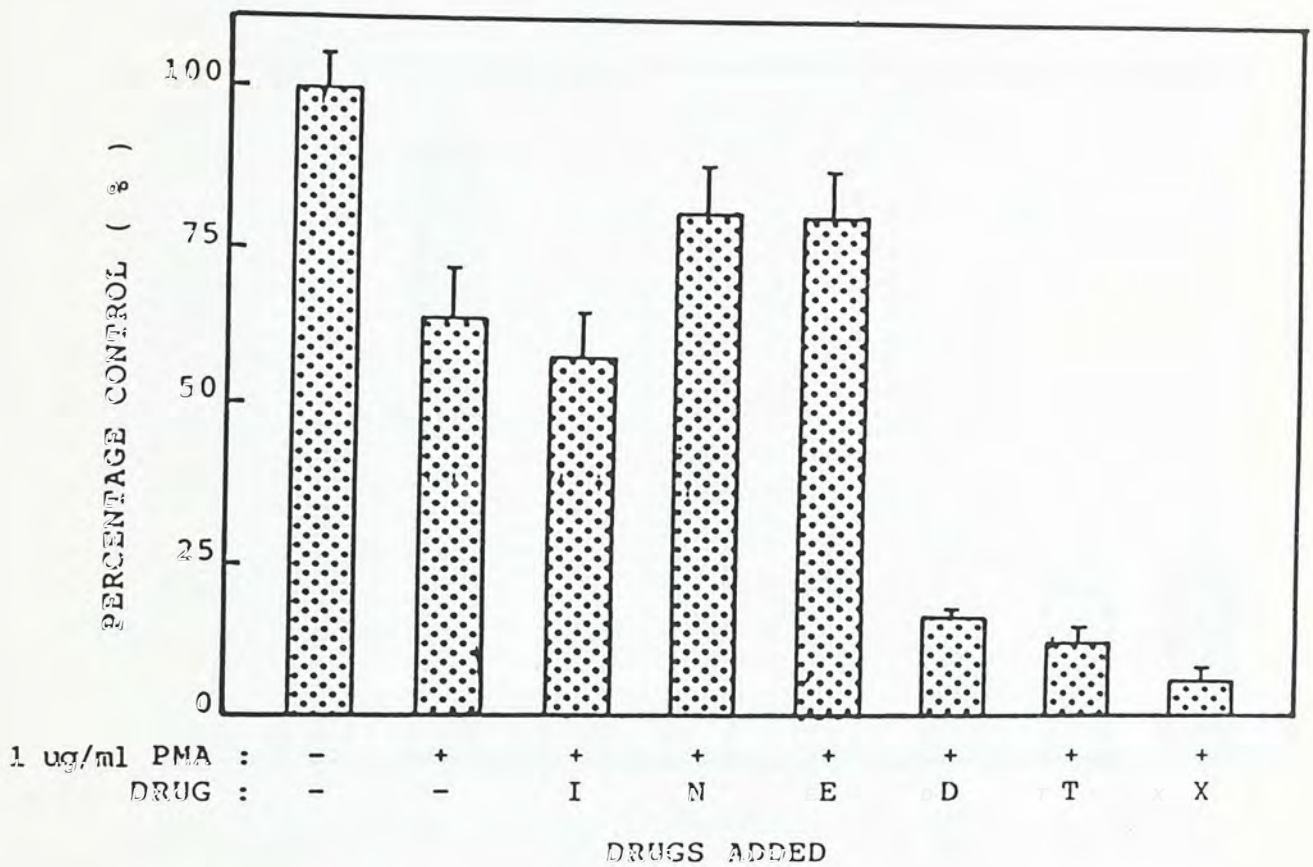

Fig. 31 Effect of various drugs on ^{3}H-thymidine incorporation in the presence of con A and PMA

3 X 10^5 rat spleen lymphocytes were cultured with various drugs as indicated above in the presence of 1 ug/ml con A in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. The 100 % control corresponded to 50,000 cpm in a typical experiment. Similar results were obtained in 2 separate experiments.
Abbreviations: I, 100 uM isoproterenol; N, 100 uM norepinephrine; E, 100 uM epinephrine; D, 100 uM dibutyryl cAMP; T, 1 mM theophylline; X, 100 uM IBMX.

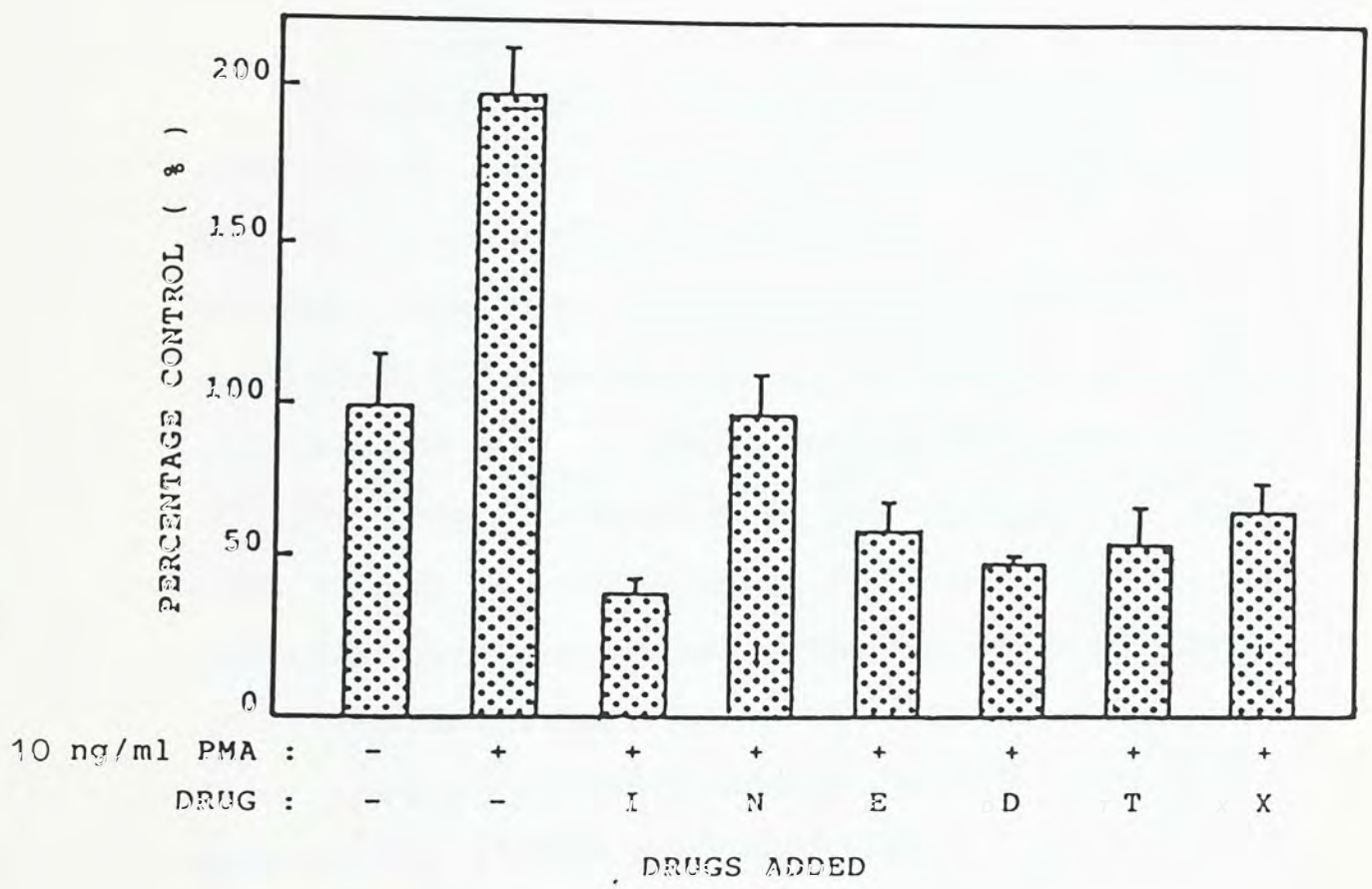

Fig. 32 Effect of various drugs on ^{3}H-thymidine incorporation in the presence of LPS and PMA

3 X 10^5 rat spleen lymphocytes were cultured with various drugs as indicated above in the presence of 10 ug/ml LPS in a flat-bottomed 96 well microtiter plate for 48 hrs at 37°C. Then 0.5 uCi ^{3}H-thymidine was introduced into each well and incubated for 6 hrs. Finally the cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. Data given are the means ± S.D. of triplicate determinations. The 100 % control corresponded to 10,000 cpm in a typical experiment. Similar results were obtained in 2 separate experiments.
Abbreviations: I, 100 uM isoproterenol; N, 100 uM norepinephrine; E, 100 uM epinephrine; D, 100 uM dibutyryl cAMP; T, 1 mM theophylline; X, 100 uM IBMX.

(Fig. 31).

3.5 Adrenergic and Cholinergic Receptor Binding on The Rat Spleen Lymphocytes

^{3}H-Dihydroalprenolol (^{3}H-DHA) is a beta-adrenergic receptor antagonist which binds to the beta receptor with high affinity. When ^{3}H-DHA (1 nM) was incubated with 4.0 x 10^{7} rat spleen lymphocyte, the total binding was about 4300 cpm and the non-specific binding in the presence of 1 mM propanolol was about 300 cpm. There were about 2.1 fmole specific ^{3}H-DHA binding sites per 10^{6} cells (Table 4). Propanolol was able to displace the specific ^{3}H-DHA binding in a dose-dependent manner with an IC_{50} of 1 uM, indicating the existence of beta-adrenergic receptors on the rat spleen lymphocytes (Fig. 33). On the other hand, little or no alpha adrenergic receptors could be detected using ^{3}H-para-aminoclonidine (^{3}H-PAC) and ^{3}H-WB4101 (Table 4) as radioligands for $alpha_2$ and $alpha_1$ adrenergic receptors respectively.

1-Quinuclidinyl-(phenyl-4-^{3}H)-benzilate (^{3}H-QNB) is a muscarinic cholinergic receptor antagonist which binds specifically to the muscarinic cholinergic receptors. When ^{3}H-QNB (2 nM) was incubated with 4.0 x 10^{7} rat spleen lymphocytes, the total binding was about 2000 cpm and the non-specific binding in the presence of 1 mM atropine was about 300 cpm. It was estimated that there were about 1.3 fmole specific ^{3}H-QNB binding sites per

Table 4 Radioligand binding on rat spleen lymphocytes

Radioligand	Total binding fmole/10^6cells	Non-specific binding fmole/10^6cells	Specific binding fmole/10^6cells
^{3}H-DHA	2.2±0.02	0.1±0.01	2.1±0.02
^{3}H-WB4101	1.1±0.45	1.2±0.17	not detectable
^{3}H-PAC	0.3±0.02	0.3±0.02	not detectable
^{3}H-QNB	1.5±0.04	0.2±0.00	1.3±0.04

Data given are the means ± S.D. of duplicate determinations.
Similar results were obtained in two separate experiments.

The results were calculated from the following equation :

$$\frac{cpm \times 10^6}{0.4 \times (2.2 \times 10^{12}) \times (4 \times 10^7) \times S} \quad \text{binding sites/}10^6\text{cells}$$

$$= 2.84 \times 10^{-14} \times \frac{cpm}{S} \times 10^{15} \quad \text{fmole/}10^6\text{cells}$$

$$= 28.4 \times \frac{cpm}{S} \quad \text{fmole/}10^6\text{cells}$$

,in which :
cpm is the counts per minute obtained by liquid scintillation counting.
10^6 is used to represent the binding sites at 10^6 cells.
0.4 is the counting efficiency.
2.2×10^{12} is the disintegrations per min for 1 Ci.
4×10^7 is the cell number at which the assay was performed.
S is the specific activity of each radioligand in Ci/mole.

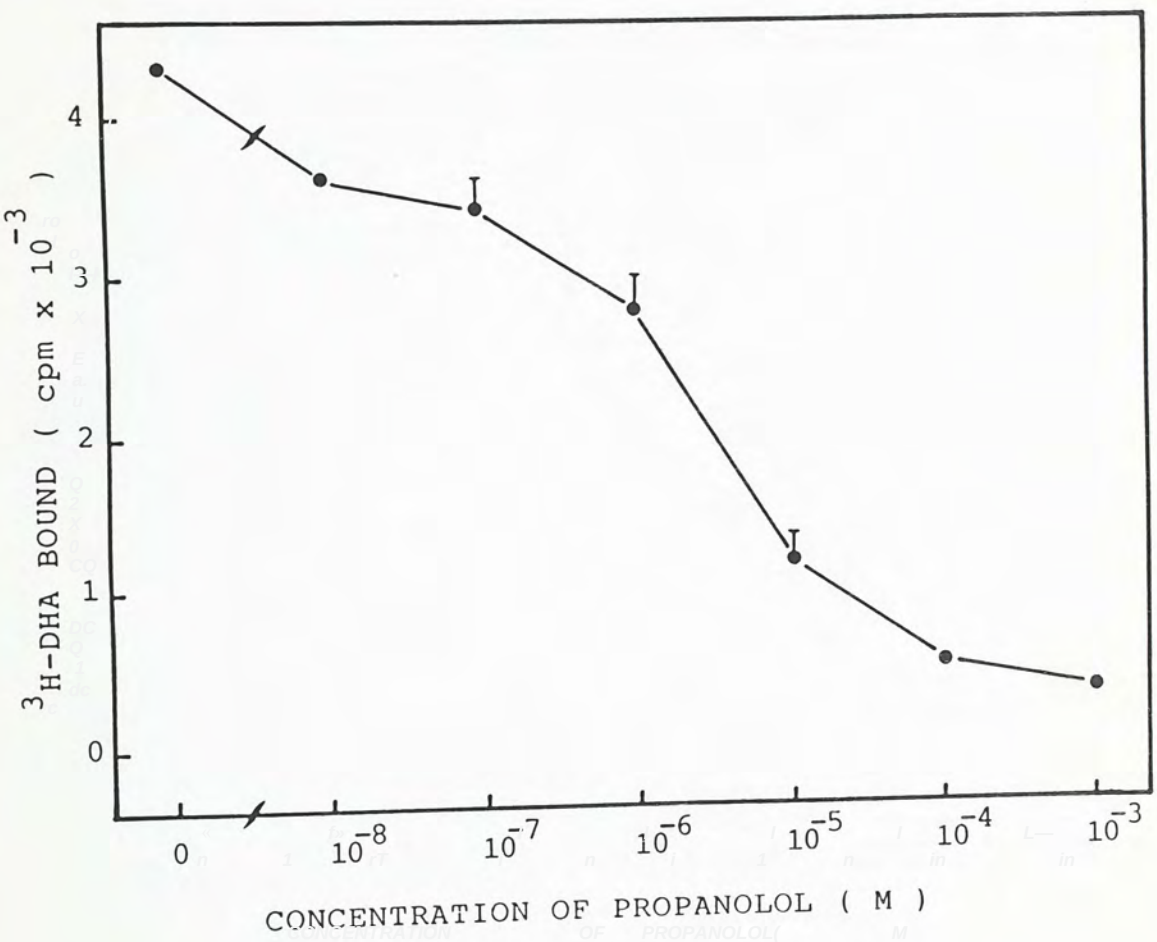

Fig. 33 Displacement curve of ^{3}H-DHA binding on rat spleen lymphocytes

4.0×10^7 rat spleen lymphocytes were incubated in the presence of ^{3}H-DHA (1 nM) and various concentrations of propanolol for 30 min at 37°C. At the end of the incubation, the cell suspension was filtered through Whatman GF/B glass fiber filter under suction. The radioactivity retained on the filter was determined by liquid scintillation counting. Data given are the means ± S.D. of duplicate determinations. Similar results were obtained in 2 separate experiments.

10^6 cells (Table 4). Atropine, which is a muscarinic antagonist, was able to displace the specific ^{3}H-QNB binding in a dose-dependent manner with an IC_{50} of 3 uM, indicating the existence of muscarinic cholinergic receptors on the rat spleen lymphocytes (Fig. 34).

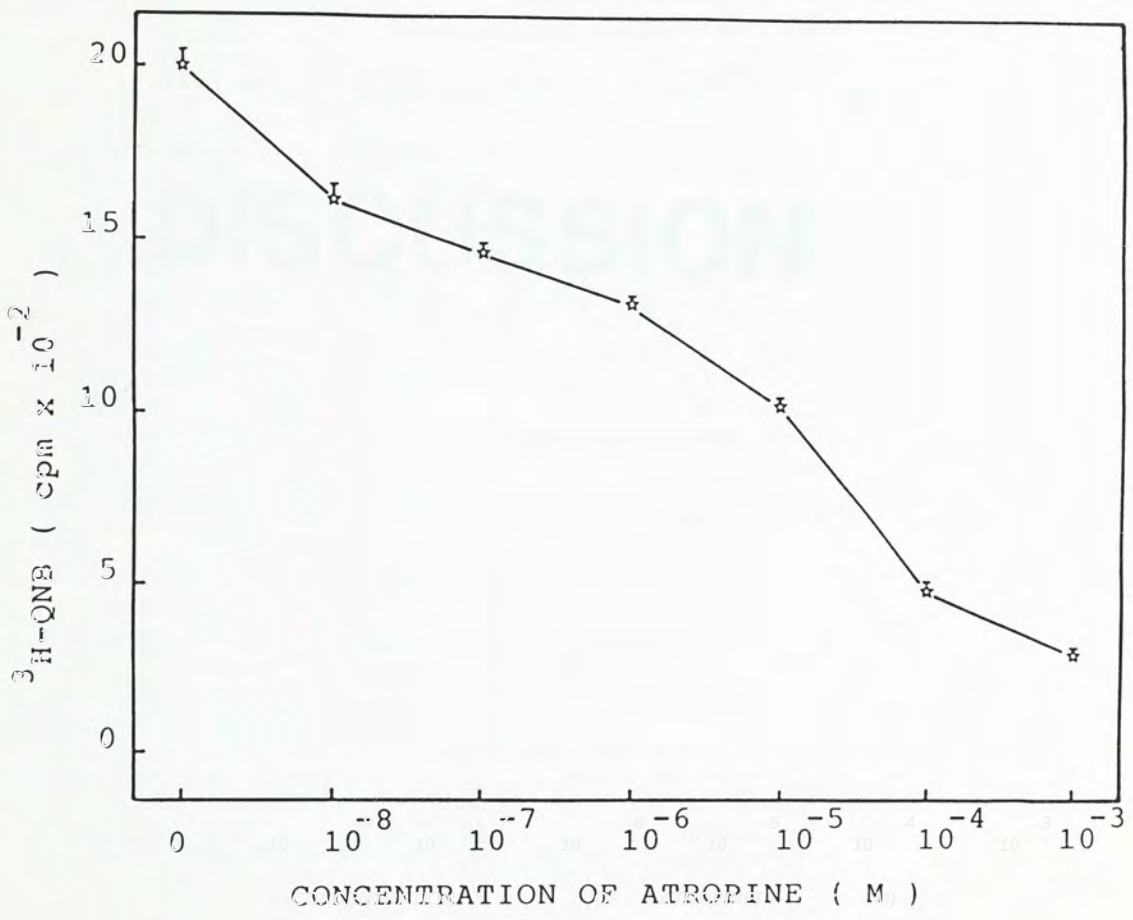

Fig. 34 Displacement curve of ^{3}H-QNB binding on rat spleen lymphocytes

4.0×10^7 rat spleen lymphocytes were incubated in the presence of ^{3}H-QNB (2 nM) and various concentrations of atropine for 60 min at 37°C. At the end of the incubation, the cell suspension was filtered through Whatman GF/B glass fiber filter under suction. The radioactivity retained on the filter was determined by liquid scintillation counting. Data given are the means ± S.D. of duplicate determinations. Similar results were obtained in 2 separate experiments.

CHAPTER FOUR

DISCUSSION

4.1 Some Characteristics of The Lymphocyte Culture System

Since lymphocyte proliferation is always accompanied by DNA replication which incorporates thymidine, it should therefore be reflected by the amount of ^{3}H-thymidine incorporated in our experiments. This assay of DNA synthesis is a sensitive one with a low background because active DNA synthesizing cells are rare in many lymphocyte populations (Ling and Kay, 1975d) and thymidine incorporation is confined to cells in the S phase (Cooper et al., 1963). Moreover, the trapping of free ^{3}H-thymidine onto the filter paper was negligible. ^{3}H-thymidine was used instead of ^{3}H-uridine or ^{3}H-leucine because activation of lymphocytes to RNA and protein synthesis is not necessarily an indication of lymphocyte proliferation e.g. while a high proportion of lymphocytes responds to PHA by an increase in RNA and protein synthesis, only half of the population actually initiates DNA synthesis (Ling and Kay, 1975d).

In order to avoid antigenic stimulation, one single spleen was used in every individual experiment. This might, however, exaggerated the variations in our experiments due to individual differences. It is fully aware that the spleen lymphocytes used in the present experiments comprised both T and B lymphocytes, and there might be the problem of interactions among the lymphocytes as well as between lymphocytes and other cell types. Future experiments should also be performed on isolated T and B lymphocyte populations in order to resolve these possibilities.

Con A, instead of PHA which is another commonly used T lymphocyte mitogen, was used because it is the best characterized plant lectin, more potent and produces less agglutination and cell damage (Stobo et al., 1972; Ling and Kay, 1975a,1975c). The dose response curve of con A shown in Fig. 1, therefore, reflected the response of T lymphocytes to the mitogen. Con A exhibited an optimal stimulation at 1 ug/ml but at higher concentrations, the response diminished. This is consistent with the results of Kaever and Resch (1985) who suggested that the decreased response was the result of an increase in intracellular cAMP (see Introduction 1.3.2).

Although LPS is a B lymphocyte mitogen, it stimulates about only 22 % of the total B lymphocyte population in nude murine spleen cultures (Watson, 1976). The dose response curve of LPS in Fig. 2 might thus only be that of a B lymphocyte subpopulation. Nevertheless, LPS was still employed since other common B lymphocyte mitogens like dextran sulphate and polyinosinic:polycytidylic acid elicited less than 10 % of the cells to respond (Watson, 1976).

An important parameter that needs to be characterized in a culture system is an optimal cell concentration so that neither the nutrients present nor the metabolites released are limiting factors to their growth. On the basis of the results presented in Fig. 4 and 5, a concentration of 3×10^5 cells per well in the

presence of either con A or LPS which give 30-50 % of the maximal response was chosen. A higher cell concentration, 10^6 cells per well, was deliberately used to increase the signal in the absence of con A or LPS so that the effects of drugs could be observed more clearly in subsequent experiments.

Both con A and LPS activated spleen lymphocytes showed maximal mitogenic index after 48 hours of incubation (Fig. 7 and 8) which was consistent with that described by Ling and Kay (1975d). Forty-eight hours of incubation was thus allowed for the lymphocytes to enter the S phase before pulse labelling of ^{3}H-thymidine. At 24 and 72 hours, increased mitogenesis was also observed indicating a heterogeneity in the periods of cell cycle among the lymphocytes (see Introduction 1.2.5). Since mitosis and cytoplasmic division can only occur after the S phase, the appearance of cell aggregates within 10 hours of incubation in the presence of con A might simply be due to the agglutinating property of con A but not cell division.

In the absence of mitogens, the ^{3}H-thymidine incorporation was quite stable except an initial drop during the first 12 hours of incubation. This probably represents a drop in cell viability plus a completion of the S phase of lymphocytes which had been activated <u>in vivo</u> before the spleen was removed. In fact, ^{3}H-thymidine incorporation in the absence of added mitogens has been suggested to be due to a continuation of proliferation activity when they are freshly prepared, and a response to stimulants in the medium like fetal calf serum (Ling and Kay, 1975c).

Therefore, ^{3}H-thymidine incorporation which occurred in the absence of mitogens should better be referred to as "spontaneous" incorporation rather than "resting" incorporation.

From the above experiments, I have established the optimal conditions for examining ^{3}H-thymidine incorporation in cultured rat spleen lymphocytes, being, 3×10^{5} cells per well in the presence of either 1 ug/ml con A or 10 ug/ml LPS or 10^{6} cells per well in the absence of mitogens, to be incubated at 37°C for 48 hours followed by 6 hours pulse labelling of ^{3}H-thymidine.

4.2 Effects of Adrenergic and Cholinergic Drugs on ^{3}H-Thymidine Incorporation

All three adrenergic drugs tested were able to suppress ^{3}H-thymidine incorporation into the rat spleen lymphocytes (Fig. 9). It appeared that both T and B lymphocytes were susceptible as both con A and LPS stimulated ^{3}H-thymidine incorporation were also inhibited (Fig. 10 and 11). Viability test by trypan blue dye exclusion indicated that these adrenergic drugs were not cytotoxic at 100 uM (Table 1). Thus, their effects on ^{3}H-thymidine incorporation cannot be accounted for by a nonspecific killing of lymphocytes. On the other hand, it is debatable whether these suppressive effects are physiologically or pharmacologically relevant. The apparent high concentration of

adrenergic drugs required for inhibiting ^{3}H-thymidine incorporation may be related to the metabolism and/or degradation of the drugs during the 48 hours of incubation at 37 °C. In fact, the adrenergic drugs underwent chemical oxidation and changed from a colorless solution to dark brown color after incubated at 37 °C for 48 hours with or without the presence of lymphocytes.

According to the *in vitro* observations presented here, the inhibitory effect of norepinephrine on the production of antibody forming cells (Besedovsky *et al.*, 1981) may be due to its inhibition of lymphocyte proliferation.

When the spleen lymphocytes were exposed to the adrenergic drugs at later times after incubation, they were less affected with respect to the ^{3}H-thymidine incorporation (Fig.15 to 17). The drugs were almost ineffective when added at 48 hours at which the lymphocytes were in the S phase (see Discussion 4.1). This is in agreement with Whitfield and co-workers (1985) who suggest that cells are most sensitive to signals in their environment during the prereplicative period to avoid the disruption and death which would result from being stopped in the middle of the S phase or mitosis. On the other hand, it can also be inferred that only a long period of exposure of lymphocytes to the adrenergic drugs could result in an appreciable suppression. In order to take such a possibility into consideration, after the addition of the adrenergic drugs at different time points, the drugs should be washed away subsequently so that the length of

drug exposure are the same.

To examine the existence of adrenergic receptors on the lymphocyte surface by radioreceptor assays, possible interference from erythrocytes which contain adrenergic receptors (Charness et al., 1976) has to be avoided. Erythrocytes in the spleen cell preparation were first lysed with Tris-buffered ammonium chloride solution and removed by washing twice in RPMI 1640 medium.

Lefkowitz (1975) suggested that 1 uM propanolol is able to fill all beta-adrenergic receptor sites even in the presence of high concentrations of radioligand. As shown in Fig. 33, it is interesting to note that propanolol as high as 100 uM was required to displace presumably all the specific ^{3}H-DHA binding on the rat spleen lymphocytes. The reason for the low potency of propanolol to displace ^{3}H-DHA was not understood. Nevertheless, the radioreceptor binding result supports the existence of beta adrenergic receptors on rat spleen lymphocytes.

No specific binding of the alpha$_1$ receptor ligand, ^{3}H-WB4101 ,and alpha$_2$ receptor ligand, ^{3}H-PAC could be detected on the rat spleen lymphocytes. These results support the suggestion that it is the beta rather than alpha receptors which are responsible for the mediation of adrenergic suppression of ^{3}H-thymidine incorporation in rat spleen lymphocytes.

Besides beta adrenergic receptors, muscarinic cholinergic

receptors were also detected on the rat spleen lymphocytes (Fig. 34). Muscarinic cholinergic binding sites had been detected on erythrocytes (Mantione and Hanin, 1980). Their interference was again eliminated in the present study by the use of the Tris-buffered ammonium chloride solution. On the other hand, since intact viable cells were used, ^{3}H-QNB might be taken up and bound intracellularly (Adem et al., 1986). The muscarinic receptor number estimated might thus be higher than the actual value. Gossuin's group (1984) claimed that such problem was not detectable when ^{3}H-methylscopolamine was used as a ligand. This makes the use ^{3}H-methylscopolamine more appropriate for labelling cell surface muscarinic receptors in the future.

Since the two biochemical reactions mediated by muscarinic cholinergic activation are either a decrease in cAMP level or an increase in phosphatidylinositol 4',5'-bisphosphate hydrolysis (Harden et al., 1986), muscarinic cholinergic activation are expected to stimulate lymphocyte proliferation (see Introduction 1.3 and 1.4, and Discussion 4.3 and 4.4). In fact, muscarinic cholinergic activation has been found to stimulate lymphocyte proliferation (see Introduction 1.1.3 abd 1.1.4). Surprisingly, both the basal and LPS-induced ^{3}H-thymidine incorporation were slightly inhibited by carbachol (Fig. 12 and 14) while the con A-induced one was unaffected (Fig. 13). None of these exhibited a stimulatory response. Therefore, it is interesting to find out if the inhibitory effects of carbachol are mediated by mechanisms other than muscarinic cholinergic activation, say, a nicotinic activation.

4.3 Effect of cAMP on ^{3}H-Thymidine Incorporation

To examine the role of cAMP in the inhibition of ^{3}H-thymidine incorporation in both T and B lymphocytes by adrenergic drugs, the effects of dibutyryl cAMP, theophylline and IBMX were examined. Dibutyryl cAMP is a cAMP derivative and is used instead of cAMP because it can pass into the cell readily while it is more difficult for the charged cAMP to enter the cell. Furthermore, cAMP is metabolized more rapidly in lymphocyte culture (MacManus et al., 1971). Theophylline and IBMX are cAMP phosphodiesterase inhibitors which can increase the intracellular cAMP level by inhibiting its degradation.

Fig. 20 to 22 showed that these cAMP elevating drugs were able to suppress the basal, con A-induced and LPS-induced ^{3}H-thymidine incorporation indicating that cAMP was indeed an inhibitory signal to lymphocyte proliferation. The fact that IBMX was more potent than theophylline in suppressing ^{3}H-thymidine incorporation as well as inhibiting cAMP phosphodiesterase suggest that cAMP accumulation was responsible for the suppression. Viability test illustrated that the suppression of ^{3}H-thymidine incorporation by dibutyryl cAMP, theophylline and IBMX was not a result of the cytotoxic effect of these agents (Table 3.3.1).

Since fetal calf serum might be responsible for the basal

^{3}H-thymidine incorporation (Ling and Kay, 1975c), cAMP might therefore be an inhibitor to the signal elicited by the serum factors (Fig. 20). Consistent with this argument is that when calf serum was depleted in 3T3 cell cultures, cAMP level rose and the cells became quiescent (Seifert and Paul, 1972). The suppression caused by the cAMP elevating agents in con A-induced ^{3}H-thymidine incorporation (Fig. 21) implied that cAMP was an inhibitory signal in T lymphocyte proliferation. In fact, it has been pointed out that the diminished proliferation caused by high con A concentrations was due to an increase in intracellular cAMP (Kaever and Resch, 1985). LPS-induced ^{3}H-thymidine incorporation has been suggested to occur as a result of inhibition of adenylate cyclase and thus a drop in the cAMP level (Jakway and DeFranco, 1986). It was therefore no surprise that cAMP elevating agents used here were able to inhibit the LPS action (Fig. 22).

Since beta adrenergic receptors had been demonstrated to be present on the lymphocyte surface (see Discussion 4.2), it was expected that beta adrenergic agonists were able to elicit cAMP production. Indeed, 1 uM isoproterenol, a beta agonist, was found to stimulate cAMP accumulation in rat spleen lymphocytes in the presence of a cAMP phosphodiesterase inhibitor, theophylline (Fig. 18 and 19). However, it was also found that the cAMP accumulation began to level off at 1 uM isoproterenol which was almost useless in inhibiting ^{3}H-thymidine incorporation (see Fig. 9-11). Such inconsistency may be accounted for by the assay system used in measuring cAMP production. For instance,

theophylline used might not effectively inhibit the phosphodiesterase activity so that high intracellular cAMP levels stimulated by high concentrations of isoproterenol could not be reached. Lysis of erythrocytes by the Tris-buffered ammonium chloride solution might perturb the integrity of lymphocytes. Incubation in the absence of energy supply might as well be a cause of the under-estimation of the EC_{50} (concentration of drug which gives 50 % excitation). Consequently, the use of more potent phosphodiesterase inhibitor, removal of erythrocytes by Ficoll gradient, and cAMP accumulation assay performed in RPMI 1640 medium without FCS which might inhibit adenylate cyclase, might perhaps yield a higher EC_{50} in eliciting cAMP accumulation. Moreover, incubation of the adrenergic drugs at 37°C for 48 hours might cause a certain degree of degradation. Thus, an apparently higher concentration was required to produce a suppression (see Discussion 4.2).

In spite of all these complications, it was still possible that adrenergic drugs, through activation of beta receptors on the lymphocyte surface, elicit cAMP accumulation which in turn suppress lymphocyte proliferation.

In the previous section, it was found that the potency of the adrenergic drugs dropped as they were added at later time points. One could argue that as the lymphocytes progressed along the cell cycle, the beta adrenergic receptors and its transduction mechanism may be progressively shut down so that adrenergic drugs in the extrace[illegible] uld not induce cAMP

accumulation inside the lymphocytes. Hence, the lymphocytes might become unresponsive. However, from Fig. 23 to 25, the cAMP elevating drugs showed a similar although not identical time dependence response in comparison to that of the adrenergic drugs. This would not only argue against the above statement but also suggest that the effect of adrenergic drugs may indeed be mediated through cAMP accumulation.

In this section, the inhibitory effect of cAMP was studied by using cAMP elevating agents. In order to further evaluate such an inhibitory effect, drugs lowering intracellular cAMP level should be explored to see if they could really stimulate ^{3}H-thymidine incorporation in the rat spleen lymphocytes.

4.4 Effects of A23187 and PMA on ^{3}H-Thymidine Incorporation

It was shown in Fig. 26 that A23187 was able to stimulate ^{3}H-thymidine incorporation in rat lymphocytes. Since A23187 is a calcium ionophore, the increase in ^{3}H-thymidine incorporation was most likely due to an increase in intracellular calcium level. There were at least three possible explanations for such an activation. Firstly, calcium ion alone might be sufficient to elicit lymphocyte proliferation. Secondly, the elevated calcium level might activate protein kinase C directly. Thirdly, the elevated calcium level might first activate a calcium dependent protease which in turn can cause a proteolytic activation of protein kinase C (Isakov et al., 1986; Murry et al., 1987). In

the last two possibilities, calcium may act synergistically with protein kinase C to trigger off DNA synthesis in the rat spleen lymphocytes. In fact, such a synergistic action had been observed in lymphocytes (Mastro and Smith, 1983). The first possibility can be differentiated from the second and third by use of polymyxin B which is a protein kinase C inhibitor. Polymyxin B should not be able to inhibit the calcium ionophore action in the first case but would inhibit in the second and third cases. Similarly, the use of protease inhibitor e.g. leupeptin (Pontremoli et al., 1986) might help to differentiate the second and third possibilities.

Another interesting phenomenon to observe was that A23187 exhibited different dose responses at the two different cell concentrations employed (Fig. 26). Such a phenomenon may possibly involve helper and suppressor factors secreted by the spleen lymphocytes. The logic is that a direct effect of A23187 could occur regardless of the cell concentration. However, only at a high enough cell concentration could the secreted soluble mediators accumulate to a high enough concentration to exert their action. At the low cell concentration (3×10^5 cells per well), 300 nM A23187 was not able to stimulate ^{3}H-thymidine incorporation. However, at the high cell concentration (10^6 cells per well), a stimulatory effect was observed. Therefore, 300 nM A23187 might not be able to activate lymphocyte proliferation directly but could activate helper T lymphocytes to secrete growth factors whose effect could be observed only at a high cell

concentration. When the A23187 concentration was raised to 1 uM, it stimulated DNA synthesis at the low cell concentration probably indicating a direct activation. However, at 1 uM A23187, a suppression was observed at the high cell concentration. This probably indicated the activation of suppressor T lymphocytes whose effect could be observed only at a high cell concentration. At 3 uM A23187, ^{3}H-thymidine incorporation in both low and high cell concentrations was suppressed. This might be due to too high an intracellular calcium level or suppression from activated suppressor T lymphocytes.

Consequently, calcium seemed to be effective in activating both helper and suppressor T lymphocytes to secrete their products besides directly stimulating lympholcyte proliferation. Moreover, suppressor factors probably were able to inhibit the action of calcium in lymphocytes.

Like DG, phorbol myristate acetate (PMA) is able to activate protein kinase C by increasing its affinity for calcium ion. Protein kinase C can then phosphorylate the Na^{+}/H^{+} exchanger to trigger off mitogenesis (Bell, 1986; Isakov et al., 1986). It was therefore no surprise that PMA could stimulate the basal (Fig. 27) ^{3}H-thymidine incorporation. However, PMA was found to inhibit the con A-induced ^{3}H-thymidine incorporation (Fig. 28). Since con A by itself can activate PIP_2 hydrolysis and thus protein kinase C activity (Sugiura and Waku, 1984), the addition of PMA might cause an overstimulation of protein kinase C activity and thus that of the Na^{+}/H^{+} exchanger. A very high intracellular pH

and Na^+ might thus be responsible for the decreased ^3H-thymidine incorporation. In the presence of LPS, PMA could stimulate ^3H-thymidine incorporation at low concentrations but inhibit at high concentrations (Fig. 29). The stimulation might be due to activation of protein kinase C while the inhibition might result from overactivation of Na^+/H^+ exchanger from protein kinase C. The use of amiloride which can inhibit the Na^+/H^+ exchanger (Rozengurt, 1986) may help to test such a hypothesis by eliminating the suppression if the Na^+/H^+ exchanger was really the cause. However, such an inhibition was not observed in the basal ^3H-thymidine incorporation. This was again possibly due to the use of a higher cell concentration (10^6 cells per well) at which helper factors released by PMA activation might accumulate to a certain level enough for stimulating proliferation.

Bijsterbosch and Klaus (1986) have shown that at high non-mitogenic concentration of con A, neither the PIP_2 hydrolysis nor the calcium ion level were decreased in comparison to those at mitogenic con A concentration. Kaever and Resch (1985) suggested that the diminished mitogenesis at high con A concentration was due to an increase in cAMP. Therefore, the inhibitory action of cAMP on lymphocyte proliferation is unlikely to be the result of an inhibition of PIP_2 hydrolysis and calcium mobilization. In fact, from Fig. 30 to 32, it could be seen that dibutyryl cAMP, theophylline and IBMX were able to inhibit ^3H-thymidine incorporation in the presence of PMA. This implies that cAMP can antagonize the action of protein kinase C in triggering

mitogenesis. The molecular events leading to DNA synthesis can be categorized into two major classes : the regulatory signals and obligatory events (Rozengurt, 1986). Regulatory signals of a given growth factor can be bypassed through the action of other regulatory factors whereas obligatory events must take place before DNA synthesis can start. Therefore cAMP might inhibit the action of protein kinase C by inhibiting : 1) the protein kinase C directly; 2) some regulatory signal(s) which act synergistically with protein kinase C to trigger off DNA synthesis; or 3) the obligatory events in DNA synthesis.

So far, it was demonstrated that LPS and PMA could increase ^{3}H-thymidine incorporation by their own. In addition, when these two drugs were added together in the lymphocyte culture, the stimulatory response was higher than the sum of the responses caused by each individual drug (Table 4.1). In other words, PMA and LPS could act synergistically in rat spleen lymphocyte proliferation. The signal mediated by LPS has been suggested to be a lowering in cAMP level (Jakway and DeFranco, 1986) while that by PMA is an increase in protein kinase C activity. It is possible that cAMP could inhibit directly the protein kinase C activity. A lowering in cAMP level might thus act synergistically with PMA to activated the protein kinase C. Alternatively, it is possible that cAMP and protein kinase C might be acting in an opposite manner on some common targets in the lymphocytes. In fact, a tyrosine specific kinase has been reported to be inhibited by the cAMP-dependent protein kinase A and stimulated by protein kinase C (Hirata et al., 1984). Activation of this

tyrosine specific kinase may play a key role in lymphocyte mitogenesis.

Since adrenergic drugs were able to produce cAMP through beta adrenergic receptors on the lymphocyte surface, they could inhibit the basal and LPS-induced ^{3}H-thymidine incorporation in the presence of PMA (Fig. 30 and 32). However, when PMA was added together with con A in the lymphocyte culture, no inhibition could be observed (Fig. 31). This indicated some kind of unresponsiveness of the lymphocytes to the adrenergic drugs. It should not be due to an unresponsiveness to cAMP because dibutyryl cAMP and the phosphodiesterase inhibitors were still inhibitory. Instead, the signal transduction mechanism might have been turned off. Whether PMA acted on the beta adrenergic receptor, guanine nucleotide binding protein or the adenylate cyclase remains to be elucidated. This phenomenon was observed only in the presence of both con A and PMA. Perhaps, only in the presence of both PMA and con A, which can also activate protein kinase C, could the protein kinase C gain a high enough activity to turn down the beta adrenergic transduction mechanism. On the other hand, interleukin 2 produced by con A activation (Mills et al., 1986), may co-operate with PMA in the desensitization of lymphocytes to adrenergic drugs.

CONCLUSION

Conclusion

Adrenergic drugs have been shown to cause a non-cytotoxic inhibition on the proliferation of rat spleen lymphocytes. The existence of beta adrenergic receptor binding and the cAMP production in response to isoproterenol suggest that such a suppression is mediated through cAMP. In fact, cAMP is shown to be inhibitory to lymphocyte proliferation by using dibutyryl cAMP and cAMP phosphodiesterase inhibitors.

Both calcium ionophore and phorbol myristate acetate (PMA), activator of protein kinase C, are able to stimulate ^{3}H-thymidine incorporation. This suggests that calcium and protein kinase C are activators of rat lymphocyte proliferation. Since an elevated intracellular calcium level and protein kinase C activity are the consequences of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, PIP_2 signalling mechanism probably exists in the rat lymphocytes.

Adrenergic drugs, dibutyryl cAMP and cAMP phosphodiesterase inhibitors can inhibit the action of PMA. Moreover, LPS, which has been suggested to lower cAMP level, can synergize with PMA in triggering mitogenesis. Therefore, activation of protein kinase C, some synergistic mechanism(s) or the obligatory events in DNA synthesis are susceptible to cAMP inhibition. Furthermore, cAMP may antagonize the action of protein kinase C on some common

target(s). A tyrosine specific kinase in lymphocytes, in fact, can be stimulated by protein kinase C but inhibited by protein kinase A. On the other hand, activation of the protein kinase C under certain circumstances may down regulate the adrenergic signal transduction mechanism. This is reflected by the unresponsiveness of lymphocytes to adrenergic drugs in the presence of both PMA and con A when dibutyryl cAMP and the cAMP phosphodiesterase inhibitors are still inhibitory.

In conclusion, both the beta adrenergic receptor linked adenylate cyclase system and the PIP_2 signalling transduction mechanism presumably exist in rat spleen lymphocytes. Activation of the former pathway leads to a decrease while activation of the latter causes an increase in lymphocyte proliferation. These two pathways thus appear to be antagonistic with each other. Moreover, cAMP has been found to inhibit the action PMA which activates protein kinase C to trigger off mitogenesis. Conversely, activation of protein kinase C may, under certain conditions, down regulate the beta adrenergic signal transduction mechanism. These observations suggest that there are strong interactions between these two pathways. It is hoped that with an understanding of the exact nature of these fundamental regulatory mechanisms and their interactions, one can find ways to manipulate the immune responses and cure some very difficult diseases as cancer (probably with depressed immune responses), acquired immunodeficiency syndrome (AIDS) (depressed immune responses), autoimmune diseases and organ transplantation (undesirably high immune responses).

REFERENCES

References :

Abell, C.W., Kamp, C.W. and Johnson, L.D. (1970) Effects of phytohemagglutinin and isoproterenol on DNA synthesis in lymphocytes from normal donors and patients with chronic lymphocytic leukemia. Cancer Res. 30:717-723.

Adem, A., Norberg, A. and Slanina, P. (1986) A muscarinic receptor type in human lymphocytes : A comparison of ^{3}H-QNB binding to intact lymphocytes and lysed lymphocyte membranes. Life Sci. 38:1359-1368.

Ader, R., Cohen, N. and Bovbjerg, D. (1983) Immunoregulation by behavioral conditioning. Trends in Pharmacol. Sci. 4:78-80.

Alquist, R.P. (1948) A study of the adrenotropic receptors. Am. J. Physiol. 153:586-600.

Andersson, J., Moller, G. and Sjoberg, O. (1972a) Selective induction of DNA synthesis in T and B lymphocytes. Cell. Immunol. 4:381-393.

Andersson, J., Sjoberg, O. and Moller, G. (1972b) Induction of immunoglobulin and antibody synthesis in vitro by lipopolysaccharides. Eur. J. Immunol. 2:349-353.

Andersson, J., Melchers, F. and Galanos, C. (1973) The mitogenic effect of lipopolysaccharide on bone marrow derived mouse lymphocytes. Lipid A as the mitogenic part of the molecule. J. Exp. Med. 137:943-953.

Averner, M.J., Brock, M.L. and Jost, J.P. (1972) Stimulation of ribonucleic acid synthesis in horse lymphocytes by exogenous cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 247:413-417.

Atweh, S.E., Grayhack, J.J. and Richman, D.P. (1984) A cholinergic receptor on muscarinic lymphocytes with novel binding characteristics. Life Sci. 35:2459-2465.

Bach, J.F. and Papiernik, M. (1981) Cellular and molecular signals in T cell differentiation. In "Microenvironments in haemopoietic and lymphoid differentiation - Ciba Foundation Symposium 84" The Pitman Press, London, pp. 215-235.

Baserga, R. (1981) The cell cycle. N. Eng. J. Med. 304:453-459.

Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices. Biochem. J. 232:211-215.

Bell, R.M. (1986) Protein kinase C activation by diacylglycerol second messengers. Cell 45:631-632.

Berridge, M.J. (1975) The interaction of cyclic nucleotides and calcium in the control of cellular activity. In "Advances in cyclic nucleotide research" (Eds : P. Greengard and G.A. Robinson) Raven Press, New York, 6:1-98.

Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220:345-360.

Berridge, M.J. (1985) The molecular basis of communication within the cell. Sci. Am. 253:124-134.

Besedovsky, H.O., del Rey, A., Sorkin, E., Da Prada, M. and Keller, H.H. (1979) Immunoregulation mediated by the sympathetic nervous system. Cell. Immunol. 48:346-355.

Besedovsky, H.O., Prada, M.D., del Rey, A. and Sorkin, E. (1981) Immunoregulation by sympathetic nervous system. Trends in Pharmacol. Sci. 2:236-239.

Bijsterbosch, M.K., Meade, C.J., Turner, G.A. and Klaus, G.G.B. (1985) B lymphocyte receptors and polyphosphoinositide degradation. Cell 41:999-1006.

Bijsterbosch, M.K. and Klaus, G.G. (1986) Concanavalin A induces Ca ++ mobilization, but only minimal inositol phospholipid breakdown in mouse B cells. J. Immunol. 137:1294-1299.

Bockoch, G.M., Katada, T., Northup, J.K., Hewlett, E.L. and Gilman, A.G. (1983) Identification of the predominant substrate for ADP-ribosylation by islet activity protein. J. Biol. Chem. 258:2072-2075.

Bourguignon, L.Y.W. and Hsing, Y.C. (1983) The participation of adenylate cyclase in lymphocyte capping. Biochim. Biophs. Acta 728:186-190.

Boynton, A.L. and Whitfield, J.F. (1983) The role of cyclic AMP in cell proliferation : A critical assessment of the evidence. In "Advances in cyclic nucleotide research" Raven Press, New York, 15:193-294.

Brown, B.L., Ekins, R.P. and Albano, J.D.M. (1972) Saturation assay for cyclic AMP using endogenous binding protein. In "Advances in cyclic nucleotide research" (Eds : P. Greengard and G.A. Robison) Raven Press, New York, 2:25-40.

Carr, M.C., Stites, D.P. and Fudenberg, H.H. (1972) Cellular immune aspects of the human fetal-maternal relationship. I. In vitro response of cord blood lymphocytes to phytohaemagglutinin. Cell. Immunol. 5:21-29.

Carrington, D.M., Auffret, A. and Hanke, D.E. (1985) Polypeptide ligation occurs during post-translational modification of concanavalin A. Nature 313:64-67.

Charness, M.E., Bylund, D.B., Beckman, B.S., Hollenberg, M.D. and Snyder, S.H. (1976) Independent variation of beta-adrenergic receptor binding and catecholamine stimulated adenylate cyclase activity in rat erythrocytes. Life Sci. 19:243-249.

Cooper, E.H., Barkhan, P. and Hale, A.J. (1963) Observation on the proliferation of human leukocytes cultured with phytohaemagglutinin. Br. J. Haematol. 9:101-111.

del Rey, A., Besedovsky, H.O., Sorkin, E., Da Prada, M. and Arrenbrecht, S. (1981) Immunoregulation mediated by the sympathetic nervous system. Cell. Immunol. 63:329.

del Rey, A., Besedovsky, H.O., Sorkin, E., Da Prada, M., and Bondiolotti, G.P. (1982) Sympathetic immunoregulation : difference between high- and low-responder animals. Am. J. Physiol. 242:R30-R33.

Delia, D., Greaves, M., Villa, S. and DeBruad, F. (1984) Characterization of the response of human thymocytes and blood lymphocytes to the synergistic mitigenicity of 12-O-tetradecanoylphorbol-13-acetate(TPA)-ionomycin. Eur. J. Immunol. 14:720-724.

Diamantstein, T. and Ulmer, A. (1975) The antagonistic action of cyclic GMP and cyclic AMP on proliferation of B and T lymphocytes. Immunology 28:113-119.

Durham, A.C. and Walton, J.M. (1982) Calcium ions and the control of proliferation in normal and cancer cells. Biosci. Rep. 2:15-30.

Felten, D.L., Felten, S.Y., Carlson, S.L., Olschowka, J.A. and Livnat, S. (1985) Noradrenergic and peptidergic innervation of lymphoid tissue. J. Immunol. 135:755s-765s.

Foker, J.E., Malkinson, A.M., Sheppard, J.R. and Wang, T. (1979) Studies of cAMP metabolism in proliferating lymphocytes. In "Molecular basis of immune cell function" (Eds: J.G. Kaplan) Elsevier, New York, pp. 57-74.

Frank, W. (1972) Cyclic 3',5' AMP and cell proliferation in cultures of embryonic rat cell. Exp. Cell Res. 71:238-241.

Gearing, A.J.H., Johnstone, A.P. and Thorpe, R. (1985) Production and assay of the interleukins. J. Immunol. Meth. 83:1-27.

Gery, I., Gershon, R.K. and Waksman, B.H.(1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. J. Exp. Med. 136:128-142.

Gillette, R.W., Mckenzie, G.O. and Swanson, M.H. (1974) Modification of the lymphocyte response to mitogens by cyclic AMP and GMP. J. Reticuloendothel. Soc. 16:289-299.

Gill, D.L., Ueda, T., Chueh, S.H. and Noel, M.W. (1986) Ca^{++} release from endoplasmic reticulum is mediated by a guanine nucleotide regulatory mechanism. Nature 320:461-464.

Gilman, A.G. (1984) G proteins and dual control of adenylate cyclase. Cell 36:577-579.

Goldstein, I.J., Hughes, R.C., Monsigny, M., Osawa, T. and Sharon, N. (1980) What should be called a lectin ? Nature 285:66.

Gossuin, A., Maloteaux, J-M., Trouet, A. and Laduron, P. (1984) Differentiation between ligand trapping into intact cells and binding on muscarinic receptors. Biochim. Biophys. Acta. 804:100-106.

Greaves, M.F. and Bauminger, S. (1972) Activation of T and B lymphocytes by insoluble phytomitogens. Nature (New Biol) 235:67.

Greaves, M. and Janossy, G. (1972) Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11:87-130.

Gunther, G.R., Wang, J.L., Yahara, I., Cunningham, B.A. and Edelman, G.M. (1973) Concanavalin A derivatives with altered biological activities. Proc. Natl. Acad. Sci. U.S.A. 70:1012-1016.

Hadden, J.W., Johnson, E.M., Hadden, E.M., Coffey, R.G. and Johnson, L.D. (1975) Cyclic GMP and lymphocyte activation. In "Immune Recognition" (Ed : A.S. Rosenthal) Academic Press, New York, pp. 359-389.

Hadden, J.W., Hadden, E.M., Sadlik, J.R. and Coffey, R.G. (1976) Effects of concanavalin A and a succinylated derivative on lymphocyte proliferation and cyclic nucleotide levels. Proc. Natl. Acad. Sci. 73:1717-1721.

Hammerling, U., Chin, A.F. and Abbott, J. (1976) Ontogeny of murine B lymphocytes : sequence of B-cell differentiation from surface-immunoglobulin-negative precursors to plasma cells. Proc. Natl. Acad. Sci. U.S.A. 73:2008-2012.

Harden, T.K., Tanner, L.I., Martin, M.W., Nakahata, N., Hughes, A.R., Helper, J.R., Evan, T., Masters, S.B. and Brown, J.H. (1986) Characteristics of two biochemical responses to stimulation of muscarinic cholinergic receptors. Trends in Pharmacol. Sci. 7:14-18.

Hawrylowicz, C.M. and Klaus, G.G.B. (1984) Activation and proliferation signals in mouse B cells. IV. Concanavalin A stimulates B cells to leave G_o , but not to proliferate. Immunology 53:703-711.

Helmreich, E.J.M. and Pfeuffer, T. (1985) Regulation of signal transduction by beta-adrenergic hormone receptors. Trends in Pharmacol. Sci. 6:438-443.

Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. and del Carmine, R. (1984) Phosphorylation at a tyrosine residue of lipomodulin in mitogen stimulated murine thymocytes. Proc. Natl. Acad. Sci. U.S.A. 81-4717-4721.

Howard, A. and Pelc, S.R. (1953) Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity Suppl. 6:261-273.

Illiano, G., Tell, G.P.E., Segel, M.I. and Cuatrecases, P. (1973) Guanosine 3',5'-cyclic monophosphate and the action of insulin and acetylcholine. Proc. Natl. Acad. Sci. U.S.A. 70:2443-2447.

Irvine, R.F., Letcher, A.J., Heslop, J.P. and Berridge, M.J. (1986) The inositol tris/tetrakis phosphate pathway-demonstration of Ins(1,4,5)P_3 3-kinase activity in animal tissues. Nature 320:631-634.

Isakov, N., Scholz, W. and Altman, A. (1986) Signal transduction and intracellular events in T-lymphocyte activation. Immunology Today 7:271-277.

Jackson, J.C., Cross, R.J., Walker, R.F., Markesbery, W.R., Brooks, WH. and Roszman, T.L. (1985) Influence of serotonin on the immune response. Immunology 54:505-512.

Jakway, J.P. and DeFranco, A.L. (1986) Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide . Science 234:743-746.

Janossy, G, Greaves, M.F.and Doenhoff, M.J. (1973) Lymphocyte activation. V. Quantitation of the proliferative responses to mitogens using defined T and B cell populations. Clin. Exp. Immunol. 14:581-596.

Johnson, H.M. and Torres, B.A. (1985) Regulation of lymphokine production by arginine vasopressin and oxytocin : Modulation of lymphocyte function by neurohypophyseal hormones. J. Immunol. 135:773s-775s.

Johnson, L.D. and Abell, C.W. (1970) The effects of isoproterenol and cyclic adenosine 3',5'-phosphate on phytohemagglutinin-stimulated DNA synthesis in lymphocytes obtained from patients with chronic lymphocytic leukemia. Cancer Res. 30:2718-2723.

Jotereau, F.V., Houssaint, E. and Le Douarin, N. (1980) Lymphoid stem cells homing to the early thymic primordium of the avian embryo. Eur. J. Immunol. 10:620-627.

Kaever, V. and Resch, K. (1985) Are cyclic nucleotides involved in the initiation of mitogenic activation of human lymphocytes? Biochim. Biophys. Acta 846:216-225.

Kolata, G. (1983) Clues to cell growth and differentiation. Science 220:291-292.

Krishnaraj, R. and Talwar, G.P. (1973) Role of cyclic AMP in mitogen induced transformation of human peripheral leukocytes. J. Immunol. 111:1010-1017.

Krug, U., Krug, F. and Cuatrecasas, P. (1972) Emergence of insulin receptors on human lymphocytes during in vitro transformation. Proc. Natl. Acad. Sci. U.S.A. 69:2604-2608.

Kurland, J.I. and Bockman, R. (1978) Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. J. Exp. Med. 147:952-957.

Lefkowitz, R.J. (1975) Technical points for using (-) ^{3}H-dihydroalprenolol in the study of beta-adrenergic receptors. In "Receptor site analysis". New England Nuclear, Boston, pp 15.

Ling, N.R. and Kay, J.E. (1975a) The activation of human and animal lymphocytes by common stimulants. In "Lymphocyte stimulation" North-Holland Publishing Company, Amsterdam, pp. 69-90.

Ling, N.R. and Kay, J.E. (1975b) Lymphocyte activating substances obtained from plants, bacteria and other sources. In "Lymphocyte stimulation" North-Holland Publishing Company, Amsterdam, pp. 103-122.

Ling, N.R. and Kay, J.E. (1975c) Response to stimulation of lymphocyte sub-populations and lymphocytes from various tissues. In "Lymphocyte stimulation" North-Holland Publishing Company, Amsterdam, pp. 237-252.

Ling, N.R. and Kay, J.E. (1975d) DNA synthesis and the cell cycle. In "Lymphocyte stimulation" North-Holland Publishing Company, Amsterdam, pp. 357-378.

MacManus, J.P. and Whitfield, J.F. (1970) Stimulation of DNA synthesis and mitotic activity of thymic lymphocytes by cyclic adenosine 3',5'-monophosphate. Exp. Cell Res. 58:188-191.

MacManus, J.P., Whitfield, J.F. and Braceland, B. (1971) The metabolism of exogenous cyclic AMP at low concentrations by thymic lymphocytes. Biochem. Biophys. Res. Comm. 42:503-509.

Male, D. (1986) Mechanism of cell cooperation. In "Immunology : An illustrated outline". Gower, London, pp. 43-44.

Mantione, C.R. and Hanin, I. (1980) Further characterization of human red blood cell membrane cholinergic receptors. Mol. Pharmacol. 18:28-32.

Marshall, W.H. and Roberts, K.B. (1965) Continuous cinematography of human lymphocytes cultured with PHA, including observations on cell division and interphase. Quart. J. Exptl. Physiol. 50:361-374.

Marx, J.L. (1984) Oncogene linked to cell regulatory system. Science 226:527-528.

Marx, J.L. (1985) The immune system "belongs in the body". Science 227:1109-1192.

Mastro, A.M. and Smith, M.C. (1982) Two signal, calcium-dependent lymphocyte activation by an ionophore, A23187, and a comitogenic phorbol ester, TPA. J. Cell. Biol. 95:21a.

Mastro, A.M. and Smith, M.C. (1983) Calcium-dependent activation of lymphocytes by ionophore, A23187, and a phorbol ester tumour promoter. J. Cell. Physiol. 116:51-56.

Mayer, S.E. (1980) Neurohumoral transmission and the autonomic nervous system. In "The pharmacological basis of therapeutics" (Eds : A.G. Gilman, L.S. Goodman and A. Gilman) Macmillan, New York, pp. 56-90.

Metcalfe, J.C., Pozzan, T., Smith, G.A. and Hesketh, T.R. (1980) A calcium hypothesis for the control of cell growth. Biochem. Soc. Symp. 45:1-26.

Mills, G.B., Stewart, D.J., Mellors, A. and Gelfand, E.W. (1986) Interleukin 2 does not induce phosphatidylinositol hydrolysis in activated T cells. J. Immunol. 136:3019-3024.

Millis, A.J.T., Forrest, G. and Pious, D.A. (1972) Cyclic AMP in cultured human lymphoid cells : relationship to mitosis. Biochem. Biophys. Res. Commun. 49:1645-1649.

Minneman, K.P., Pittman, R.N. and Molinoff, P.B. (1981) Beta-adrenergic receptor subtypes : properties, distribution, and regulation. Ann. Rev. Neurosci. 4:419-461.

Moolenaar, W.H., Boonstra, J., van der Saag, P.T. and de Laat, S.W. (1981) Sodium/proton exchange in mouse neuroblastoma cells. J. Biol. Chem. 256:12883-12887.

Moolenaar, W.H., Yarden, Y., de Laat, S.W. and Schlessinger, J. (1982) Epidermal growth factor induces electrically silent Na influx in human fibroblasts. J. Biol. Chem. 257:8502-8506.

Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. and de Laat, S.W. (1983) Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature 304:645-648.

Muallem, S., Schoeffield, M., Pandol, S. and Sachs, G. (1985) Inositol trisphosphate modification of ion transport in rough endoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A. 82:4433-4437.

Murray, A.W., Fournier, A. and Hardy, S.J. (1987) Proteolytic activation of protein kinase C : a physiological reaction ? Trends in Pharmacol. Sci. 12:53-54.

Nishizuka, Y. (1984a) Turnover of inositol phospholipids and signal transduction. Science 225:1365-1370.

Nishizuka, Y. (1984b) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693-698.

Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U. and Kaibuchi, K. (1984) Phospholipid turnover in hormone action. Recent Progress in Hormone Research 40:301-345.

Novogrodsky, A. and Katchalski, E. (1971) Lymphocyte transformation induced by concanavalin A and its reversion by methyl-alpha-D-mannopyranoside. Biochim. Biophys. Acta 228:579-583.

O'Dorisio, M.S., Wood, C.L. and O'Dorisio, T.M. (1985) Vasoactive intestinal peptide and neuropeptide modulation of the immune response. J. Immunol. 135:792s-796s.

Oppenheim, J.J. (1969) Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. Fed. Proc. Fed. Am. Soc. Exp. Biol. 27:21-28.

Otten, J., Johnson, G.S. and Pastan, I. (1922) Regulation of cell growth by cyclic adenosine 3',5'-monophosphate : Effect of cell density and agents which alter cell growth on cyclic adenosine 3',5'-monophosphate levels in fibroblasts. J. Biol. Chem. 247:7082-7087.

Payan, D.G. and Goetzl, E.J. (1985) Modulation of lymphocyte function by sensory neuropeptides. J. Immunol. 135:783s-786s.

Peavy, D.L., Shands, J.W.Jr. and Adler, W.H. (1973) Mitogenicity of bacterial endotoxins : characterization of the mitogenic principle. J. Immunol. 111:352-357.

Philip, H.J. (1973) Dye exclusion tests for cell viability. In "Tissue culture : methods and applications" (Eds. Kruse and Patterson) New York, Academic Press, pp. 406.

Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986) Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca^{++} -requiring proteinase. J. Biol. Chem. 261:8309-8311.

Ranelletti, F.O., Maggiano, N., Aiello, F.B., Carbone, A., Larocca, L.M., Musiani, P. and Piantelli, M. (1987) Glucocorticoid receptors and corticosensitivity of human thymocytes at discrete stages of intrathymic differentiation. J. Immunol. 138:440-445.

Richman, D.P. and Arnason, B.G.W. (1979) Nicotinic acetylcholine receptor : Evidence for a functionally distinct receptor on human lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 76: 4632-4635.

Rixon, R.H., Whitfield, J.F. and MacManus, J.P. (1970) Stimulation of mitotic activity in rat bone marrow and thymus by exogenous adenosine 3',5'-monophosphate (Cyclic AMP). Exp. Cell Res. 63:110-116.

Robb, R.J. (1984) Interleukin 2 : the molecule and its function. Immunology Today 5:203-209.

Roitt, I.M., Brostoff, J. and Male, D. (1985) Lymphoid cells. In "Immunology" Gower, London, pp 2.1-2.7.

Rosenstreich, D.L., Nowtny, A. and Chused, T. (1973) In vitro transformation of mouse bone-marrow-derived (B) lymphocytes induced by the lipid component of endotoxin. Infect. Immun. 8:406-411.

Roszman, T.L., Jackson, F.C., Cross, R.J. and Michael, J.T. (1985) Neuroanatomic and neurotransmitter influences on immune function. J. Immunol. 135:769s-722s.

Rozengurt, E. (1986) Early signals in the mitogenic response. Science 234:161-166.

Russell, D.H., Matrisian, L., Kibler, R., Larson, D.F., Poulos, B. and Magun, B.E. (1984) Prolactin receptors on human lymphocytes and their modulation by cyclosporine. Biochem. Biophy. Res. Comm. 121(3): 899-906.

Sasaki, M.S. and Norman, A. (1966) Proliferation of human lymphocytes in culture. Nature 210:913-914.

Schreiner, G.R. and Unanue, E.R. (1975) The modulation of spontaneous and anti-Ig stimulated motility of lymphocytes by cyclic nucleotide and adrenergic and cholinergic agents. J. Immunol. 14:802-808.

Seifert, W. and Paul, D. (1972) Levels of cyclic AMP in spause and dense cultures of growing and quiescent 3T3 cells. Nature. (New Biol.) 240:281-283.

Shackelford, D.A. and Trowbridge, I.S. (1984) Induction of expression and phosphorylation of the human interleukin 2 receptor by a phorbol diester. J. Biol. Chem. 259:11706-11712.

Shapiro, H.M. and Strom, T.B. (1980) Electrophysiology of T lymphocyte cholinergic receptors. Proc. Natl. Acad. Sci. U.S.A. 77:4317-4321.

Shenkman, L., Rabey, J.M. and Gilad, G.M. (1986) Cholinergic muscarinic binding by rat lymphocytes : Effects of antagonist treatment, strain and aging. Brain Res. 380:303-308.

Smith, K.A. (1980) T-cell growth factor. Immunol. Rev. 51:337-357.

Smith, E.M., Harbour-Mcmenamin, D. and Blalock, J.E. (1985) Lymphocyte production of endorphins and endorphin-mediated immunoregulatory activity. J. Immunol. 135:779s-782s.

Snow, E.C. (1985) Insulin and growth hormone function as minor growth factors that potentiate lymphocyte activation. J. Immunol. 135:776s-778s.

Soren, L. (1973) Variability of the time at which PHA-stimulated lymphocytes initiate DNA synthesis. Exp. Cell Res. 78:201-208.

Spat, A., Bradford, P.G., Mckinney, J.S., Rubin, R.P. and Putney, J.W., Jr. (1986) A saturable receptor for ^{32}P-inositol-1,4,5-trisphosphate in hepatocytes and neutrophils. Nature 319:514-516.

Steffen, J.A. and Stolzman, W.M. (1969) Studies on in vitro lymphocyte proliferation in cultures synchronized by inhibition of DNA synthesis. I. Variability of S plus G periods of first generation cells. Exp. Cell. Res. 56:453-460.

Stobo, J.D., Rosenthal, A.J. and Paul, W.E. (1972) Functional heterogenity of murine lymphoid cells. I. Responsiveness to and surface binding of con A and PHA. J. Immunol. 108:1-17.

Strab, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Release of Ca^{++} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. Nature 306:67-69.

Strom, T.B., Sytkowski, A.J., Carpenter, C.B. and Merrill, J.P. (1974) Cholinergic augmentation of lymphocyte mediated cytotoxicity. A study of the cholinergic receptor of cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 71:1330-1333.

Strom, T.B., Hirsch, M.S., Black, P.H., Carpenter, C.B., Philips, S.M. and Merrill, J.P. (1975) Modulation of GVH proliferation by cyclic nucleotides. Transplant. Proc. 7:305.

Sugiura, T. and Waku, K. (1984) Enhanced turnover of arachidonic acid containing species of phosphatidylinositol and phosphatidic acid of concanavalin A-stimulated lymphocytes. Biochim. Biophys. Acta 796:190-198.

Sumner, J.B. and Howell, S.F. (1936) The identification of the hemaggiutinin of the jack bean with con A. J. Bacteriology 32:227.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. J. Biol. Chem. 252:7603-7609.

Taylor, C.W. (1987) Receptor regulation of Ca^{++} entry. Trends in Pharmacol. Sci. 8:79-81.

Taylor, P. (1980) Cholinergic agonists. In "The pharmacological basis of therapeutics" (Eds: G.G. Gilman, L.S. Goodman and A. Gilman) Macmillan, New York, pp. 91-99.

Van Wauwe, J.P. De Mey, J.R. and Goossens, J.G. (1980) OKT3 : a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. J. Immunol. 124:2708-2713.

Watson, J. (1975) The influence of intracellular levels of cyclic nucleotides on cell proliferation and the induction of antibody synthesis. J. Exp. Med. 141:97-111.

Watson, J. (1976) The involvement of cyclic nucleotide metabolism in the initiation of lymphocyte proliferation induced by mitogens. J. Immunol. 117:1656-1663.

Whitfield, J.F., Boynton, A.L., MacManus, J.P., Rixon, R.H., Sikorska, M., Tsang, B., Walker, P.R. and Swierenga, S.H.H. (1980) The roles of calcium and cyclic AMP in cell proliferation. Ann. N. Y. Acad. Sci. 339:216-240.

Whitfield, J.F., Alton, L.B., Rixon, R.H. and Youdale, T. (1985) The control of cell proliferation by calmodulin, Ca^{++} - calmodulin, and cyclic AMP. In "Control of Animal Cell Proliferation" (Eds : A.L. Boynton and H.L. Leffert) Academic Press, London, pp. 331-365.

Whitlock, C., Denis, K., Robertson, D. and Witte, O. (1985) In vitro analysis of murine B-cell development. Ann. Rev. Immunol. 3:213-235.

Witters, L.A., Vater, C.A. and Lienhard, G.E. (1985) Phosphorylation of the glucose transporter in vitro and in vivo by protein kinase C. Nature 315:777-778.

Xu, S.Y. (徐叔云) and Chen, M.Z. (陈毓珠) (1985) 神经递质对免疫的调控. 「药理学进展 1983-1985」(主编王振纲、宋书元). 人民卫生出版社 北京, 31-45页.

Yarvi, J., Kalb, J. and Levitzki, A. (1968) The interaction of con A with methyl-α-D-glucopyranoside. Biochim. Biophs. Acta 165:303.

Zalcman, S.J., Neckers, L.M., Kaayalp, O. and Wyatt, R.J. (1981) Muscarinic cholinergic binding sites on intact human lymphocytes. Life Sci. 29:69-73.

Zwiller, J., Revel, M-O. and Malviya, A.N. (1985) Protein kinase C catalyses phosphorylation of guanylate cyclase in vitro. J. Biol. Chem. 260:1350-1353.

Appendix :

RPMI-1640 MEDIA

FORMULATION

	COMPONENT	R 4130 (with L-glutamine & 25 mM HEPES)
INORGANIC SALTS	Calcium Nitrate $4H_2O$	0.100
	Magnesium Sulfate (Anhydrous)	0.04884
	Potassium Chloride	0.400
	Sodium Chloride	6.000
	Sodium Phosphate Dibasic (Anhydrous)	0.800
AMINO ACIDS	L-Arginine (Free Base)	0.200
	L-Asparagine (Anhydrous)	0.050
	L-Aspartic Acid	0.020
	L-Cystine 2HCl	0.0652
	L-Glutamic Acid	0.020
	L-Glutamine	0.300
	Glycine	0.010
	L-Histidine (Free Base)	0.015
	L-Hydroxyproline	0.020
	L-Isoleucine	0.050
	L-Leucine	0.050
	L-Lysine HCl	0.040
	L-Methionine	0.015
	L-Phenylalanine	0.015
	L-Proline	0.020
	L-Serine	0.030
	L-Threonine	0.020
	L-Tryptophan	0.005
	L-Tyrosine (Disodium)	0.02883
	L-Valine	0.020
VITAMINS	d-Biotin	0.0002
	Choline Chloride	0.003
	Folic Acid	0.001
	myo-Inositol	0.035
	Niacinamide	0.001
	p-Amino Benzoic Acid	0.001
	d-Pantothenic Acid (Calcium)	0.00025
	Pyridoxine HCl	0.001
	Riboflavin	0.0002
	Thiamine HCl	0.001
	Vitamin B_{12}	0.000005
OTHER	Aminopterin	—
	D-Glucose	2.000
	Glutathione Reduced	0.001
	HEPES	5.958
	Hypoxanthine	—
	Phenol Red (Sodium)	0.0053
	Thymidine	—
ADD	Sodium Bicarbonate	2.000
SPECS	pH at 25°C (without Sodium Bicarbonate)	6.7±0.2
	pH at 25°C (with Sodium Bicarbonate)	7.0±0.2
	Osmolarity—mOsm/Kg H_2O (without Sodium Bicarbonate)	265±5%
	Osmolarity—mOsm/Kg H_2O (with Sodium Bicarbonate)	288±5%